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The use of autologous adult stem cells in the treatment of multiple sclerosis; evidence for pathophysiological roles of sub-populations and clinical monitoring of cells and treatment effects.

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Abstract

Despite significant advances in the efficacy of immunotherapies that target increasingly specific inflammatory elements critical to the development of the disease, there remains a significant therapeutic shortfall for sufferers of Multiple Sclerosis (MS). The pathophysiological disconnect between inflammation and the neurodegeneration that ultimately underlines disability is well described and continues to limit the success of increasingly potent immunosuppression.

Neuroprotection, immunomodulation and cell replacement, all demonstrable properties of adult multipotent mesenchymal stem cells (MSCs) offer further therapeutic potential that may help to address both the inflammatory disease component and the neuronal loss that underscores disability. Much work has been done over the last decade and more to elucidate these properties with the accumulation of a critical body of evidence enabling translation to clinical trials including in Bristol.

Animal models of inflammatory neurological pathology have yielded many clues to the mode of activity of MSCs. Detailed knowledge of their pathophysiological role in man however is lacking.

This thesis was principally directed towards supporting and refining the imminent translational study. The aim of this work was twofold; to identify stem cell populations with proven advantageous properties in the bloodstream and in the brains of individuals with MS and interrogation of Neurophysiological outcome measures as a surrogate for disability measures.

Stem cells of the bone marrow niche were identified in patients undergoing clinical relapse, as well as in those taking commonly prescribed disease modifying therapies. Whilst MSCs were readily identified in bone marrow samples, they were not detectable in peripheral blood at current technological thresholds. Cells expressing markers commonly expressed by MSCs were also identified in post-mortem brain tissue from individuals with active MS, suggesting possible central nervous system infiltration. Finally evoked potential-based composite scores were demonstrated to correlate with clinical disability scoring methods both in cross-sectional and to a lesser extent longitudinal analysis.

Dedication and Acknowledgements

I would primarily like to acknowledge Professor Scolding whose generosity and guidance has enabled me to undertake this work. It has been a privilege to work in his laboratories.

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Jonathan Witherick

Table of Contents

Chapter 1

Introduction

Multiple Sclerosis

The earliest recordings of the disease process that has come to be called Multiple Sclerosis may be found in references to the spontaneous resolution of sight in young females in biblical teachings or Norse mythology. Examples of MS were undoubtedly contained in case series of paraplegia and limb palsy produced in early efforts at systematic descriptions of what has become known as Neurological disease. The first depiction of MS appears in 1838 in Robert Carswell's famous book *Pathological anatomy: illustrations of the elementary forms of disease.* More detailed and systematic descriptions of the disease were produced by Jean-Martin Charcot at the latter end of the 19th century. The initial terminology of *sclerose en plaque disseminees* or disseminated sclerosis was only replaced by the modern day multiple sclerosis in the mid-20th century when consensus was reached primarily through the organisation of patient support groups and then finally with publication of the first edition of McAlpine's *Multiple Sclerosis*. As the 19th century progressed progressively more light was shone on the processes underlying the disease with the advent at the turn of the century of the first effective disease-modifying therapeutic agents. Much however remains enigmatic and the therapeutic needs of people with the disease remain significantly under met.

Multiple sclerosis is the commonest cause of non-traumatic disability in young adults (Sadovnick and Ebers, 1993) with a UK prevalence of between 103-186 per 100,000 (Pugliatti et al., 2006). Worldwide, estimates put the number of affected individuals at more than 2.5 million (Compston and Coles, 2002). Costs associated with the disease are significant and proportional to the level of disability; in 2006 UK costs were estimated at £12,000 for those with an EDSS <4 and £60,000 with EDSS of 7 or more (Kobelt et al., 2006). Overall costs were not calculated in this study due to insufficient prevalence data but were estimated to be 40% higher than the £1.9 billion calculated by a Stockholm School of Economics working paper in 2000. A significant proportion of associated costs are due to increasingly expensive disease-modifying therapies.

Multiple sclerosis, as with other autoimmune disease, is more common in women. The generally agreed ratio is F:M - 2:1 though the proportion of females has varied from a maximum of 71% in some US and New Zealand case series (Miller et al., 1992; Visscher et al., 1984) to 51% in several Israeli series (Leibowitz et al., 1973). Peak age of onset is roughly 30. Though a significant proportion of patients experience onset of the disease before they are 20, it is rare in children. In this group, as with the over 50's, alternative diagnoses should be explored thoroughly. The hallmark of multiple sclerosis is the presence of demyelinated plaques in the CNS with dissemination in time and space. Clinical, biological, electrophysiological and radiological surrogates of the process are utilised in the diagnosis of the disease. Diagnostic criteria have been developed to standardise epidemiological study, therapeutic trials and, with the advent of potentially harmful DMDs, clinical

practice. The first of these were developed by Poser and colleagues (Poser et al., 1983) generating clinically and laboratory-supported definite and probable MS. The difficulties posed by primary progressive disease were first addressed by Thompson and colleagues (Thompson et al., 2000) who distinguished the entity by clinical progression for at least one year with 3 levels of diagnostic certainty, established using clinical parameters, MRI criteria, the presence of intra-thecal oligocloncal band synthesis and visual evoked potentials. Further evolution of diagnostic criteria has taken place over the last 15 years to enable earlier confident diagnosis (particularly relevant in the light of increasing therapeutic options) and to allow adaptation to accumulating evidence of the predictive value of laboratory tests. In 2001 an International Panel produced the 'McDonald criteria' (McDonald et al., 2001) unifying diagnostic criteria for both relapsing and progressive from onset disease.

The clinical course of Multiple Sclerosis is well documented. Current consensus classifies disease into one of four categories; primary progressive, relapsing-remitting, secondary progressive and relapsing progressive disease (Lublin and Reingold, 1996). 80-85% of patients experience acute, rapid onset symptoms, a 'relapse', as the first clinical sign of the disease (Confavreux et al., 1980; Poser et al., 1982) (although often, a prior episode that was either trivialised or misdiagnosed, is apparent on taking a detailed neurological history). The first episode was previously referred to as a clinically isolated syndrome; not all cases will show disease recurrence or progression to establish a diagnosis of MS. Evidence of subclinical disease activity on neuroimaging increases the likelihood of

further clinical relapses (Fisniku et al., 2008). The average rate of relapse is difficult to establish definitively due to potential mimics (including transient neuronal dysfunction due to environmental challenges to saltatory conduction in the CNS – so-called 'pseudo-relapses') and lack of a 'gold standard' confirmatory clinical test. Retrospective reporting estimates relapse rates of generally <0.5 per year (McALPINE and COMPSTON, 1952; Myhr et al., 2001) and prospective assessments at slightly higher rates of 0.5-1.0 (Confavreux et al., 1980). Rarely does average relapse frequency exceed 1.5 per year (Compston and Coles, 2008). One of the most difficult aspects to reconcile for the patient with MS is the unpredictable nature of relapses which can strike at any time. Recovery is often incomplete following each relapse and with time there is slow accumulation of disability. In time, 65% of patients will enter a progressive phase of the disease – secondary progressive MS; whilst in 15-20% the disease is progressive from onset – primary progressive disease (Confavreux et al., 1980; Poser et al., 1982). Entry into the progressive phenotype of the disease occurs at roughly the same age (40), regardless of the presence of antecedent relapses (Confavreux and Vukusic, 2006a). At present, there are no reliable biomarkers that can predict both if and when patients with relapsing disease will enter the progressive phases – this is one of many areas of current research priority. Analysis of the death of participants in longitudinal studies, whilst relatively infrequent (e.g. 16/1099 patients in the Ontario cohort) demonstrate a median time to death from onset of MS of 30 years. This figure is supported by data from a Danish study looking specifically at death rather than disease progression in which 4254/9881 participants had died (Brønnum-Hansen et al., 2004).

Aetiology of Multiple Sclerosis

Epidemiological studies in MS have provided useful insights into the genetic and environmental contribution to disease manifestation. Neither provides an explanation of the distribution of the disease in isolation. Findings point to a multifactorial aetiology with disease occurrence in genetically at-risk individuals exposed to requisite environmental conditions.

Evidence from epidemiological studies demonstrates two consistent findings; MS clusters in families (Robertson et al., 1996) and rates of MS vary widely across the globe (Simpson et al., 2011). These and more detailed subgroup findings suggest both play a role in the causation of MS; migrant studies have been particularly informative in this context. Evidence for the genetic component of MS comes from twin studies demonstrating concordance of 25% in monozygotic twins (up to 35% in female twins) (Willer et al., 2003). This and evidence from analysis of twins, adoptees and migrants point to a polygenic model with a moderate effect allele (odds ratio ~3-4) and multiple smaller effect alleles (odds ratio <1.5) (Sawcer et al., 2014). Associations between MS and variation in the major histocompatibility complex genes encoding the human leucocyte antigen were first identified in the 1970s (Naito et al., 1972). Interrogation of the allelic association of biologically plausible/relevant complexes targeted genetic research within the field of MS over subsequent decades. Early work focusing on HLA-A3 and B7 (Jersild et al., 1972) has been refined and has established the allelic association of the HLA molecules DR15 - with DRB1*1501 (the main risk allele for MS) and DRB5*0101, and DQ6 -

with DQA1*0102 and DQB2*0602. Alongside this work, linkage of genes within the MHC complex and susceptibility to MS has been interrogated, and in some commentator's eyes resolved, through studies analysing large numbers of affected families (Ebers et al., 1996; Sawcer et al., 1996). Prior to the advent of whole genome association studies little progress was made in identifying risk alleles out with the MHC. The largest genome-wide association study thus far has identified 57 single nucleotide polymorphism (SNP) associations (Sawcer et al., 2011) with a further 2 added in subsequent studies (Lill et al., 2013; Patsopoulos et al., 2011). The identified SNPs are notable for a high proportion with an association with immune system function, particularly in the domain of T cell differentiation. Broadly speaking candidate genes fall into one of several different categories including; cytokines involved in immune cell interaction, chemokines, cell adhesion molecules, inflammatory mediators and growth factors.

Evidence suggests that geographical variation is explained by a combination of genetic and environmental factors. A latitudinal gradient with higher rates of MS being associated with increasing distance from the equator (Simpson et al., 2011) has been established. Some of the inconsistencies in this somewhat simplified model can be explained by correction for genetic factors/risk alleles. The most likely environmental factor explaining the regional variation is levels of UV light and vitamin D synthesis in native populations. Analysis of migrant populations affords significant clues to the interplay between genetic and environmental factors. Populations in Australia migrated from Europe have gradients of frequency that do not follow genetic inheritance patterns but remain correlated with latitude

(Hammond et al., 1988). Furthermore, the rates of MS in immigrant populations demonstrates that those that move before age 15 adopt the incidence of the location to which they move whereas if moving after 15 they retain the risks of the population from which they emigrated (Dean, 1967). Some have attempted to correct for age and sex and found that the north-south gradient for age and sex-adjusted prevalence diminishes and age-adjusted incidence disappears completely, suggesting that distribution may be an artefact of demographic differences (Zivadinov et al., 2003), though this is contended.

Environmental triggers have been hypothesised for some time but systematic evidence is lacking. Putative candidates have included both infective and non-infective agents. Work in Sweden (Lindberg et al., 1991) and the UK (Martyn et al., 1993) suggested an increased relative risk of developing MS in the context of prior Epstein Barr Virus (EBV) infection. Further evidence implicating EBV in the aetiology and development of MS comes from immunological studies demonstrating molecular mimicry by the virus of Myelin Basic Protein (MBP) epitopes (Lang et al., 2002). Other potential agents that have attracted significant attention include Human Herpes Virus 6 (HHV6) (Ablashi et al., 1998; Chapenko et al., 2003) and Chlamydia pneumonia (Sriram et al., 1999) though evidence is contradictory and candidature insecure. Various non-infectious agents have been postulated to play a role in the development of MS including parity, exogenous hormones, trauma and mercury in amalgam dental fillings, though none have gained any traction.

Pathology of Multiple Sclerosis

The pathological findings in MS are central to the disease and its description – indeed even its name. The central underlying disease process is inflammation leading to the destruction of myelin sheaths and demyelination. This is the essential lesion, occurring throughout the CNS with particular prevalence for periventricular white matter, the spinal cord and optic nerves. The process is accompanied by astrocytosis and scar formation resulting in the visible plaques that give the condition its name.

Pathological description of the disease began with the observations of Carswell in 1838 of multiple discoloured and indurated lesions throughout the CNS (Carswell, 1838). Correlation with a clinical phenotype was lacking other than a description of simply 'paralysis'. The illustrations were based on 2 French cases in whom Carswell records 'I could not ascertain that there was anything in the character of the paralysis or the history of the cases calculated to throw any light on the nature of the lesion found in the spinal cord'. There is however little doubt that the lesions represented are the first identification of the pathology of MS. Later in the 19th century, and still at a remarkably early stage, Eduard Rindfleisch made several observations of some of the core pathological features of the condition including loss of myelin, formation of scar tissue and the orientation of lesions around medium-sized blood vessels (Rindfleisch, 1863). The first systematic pathological description was made by Charcot in 1868 (Charcot, 1868) and has been regularly expanded ever since.

Classical Multiple Sclerosis is only one of several different types of demyelinating diseases that affect the human nervous system. There is evidence that this apparently clinically disparate group of conditions causing widely varying phenotypes do in fact share very similar pathogenetic mechanisms. Cases in which various combinations coexist have occasionally been reported. Furthermore the entire spectrum of human inflammatory disease can be reproduced in experimental models of inflammatory disease simply by using different methods of immuno-stimulation (Lassmann, 1983). These include;

- acute or Marburg variant MS (Marburg, 1906) in which there are particularly destructive lesions spread throughout the central and peripheral nervous system
- concentric sclerosis of Balo (Balo, 1928) in which unknown mechanisms cause alternating rims of myelin preservation and loss leading to the typical onion bulb lesions
- neuromyelitis optica caused by antibodies to a transmembrane protein (aquaporin 4) found in high concentration in the spinal cord and optic nerve (Devic, 1894)
- acute demyelinating encephalomyelitis and transverse myelitis; a monophasic condition often associated with prior vaccination or infection, with more extreme cases resulting in perivascular haemorrhage and brain oedema (acute haemorrhagic leukoencephalomyelitis)
- acute (Guillain et al., 1916) and chronic (Albers and Kelly, 1989) forms of inflammatory demyelinating peripheral polyneuropathy in which there is demyelination of peripheral nerves and in severe cases axonopathy

In the classical plaque of multiple sclerosis axons are demyelinated but preserved in an astrocytic scar matrix. Plaques are centred on medium sized vessels (Rindfleisch, 1863) with prevalence in periventricular white matter and the outer surface of the brain (Steiner, 1931) and in the spinal cord (Oppenheimer, 1978). Histological specimens can only ever provide snapshots of a dynamic disease process. Lesions or plaques can be broadly classified as active, chronic active and inactive.

In actively demyelinating lesions myelin degradation products are identified within infiltrating macrophages which are abundant (Brück et al., 1995; Gay et al., 1997). Chronic active lesions are characterised by a rim of activated microglia and active demyelination at the periphery of the lesion, surrounding a central area of completed demyelination. In the later stages of disease, when there is progressive clinical deterioration, it is unclear whether microscopic areas of ongoing active inflammation represent an active disease process or a response to secondary Wallerian degeneration. The progression of an active lesion to inactivity is not of course binary. Studies have identified the persistence of macrophages with myelin degradation products for months in Wallerian degeneration (Lumsden, 1970), and the presence of free lipids on MR spectroscopic analysis for several months after lesion initiation (Davie et al., 1994). In so-called destructive lesions, there is extensive additional tissue destruction affecting both astrocytes and axons – seen in severe and rapidly progressive disease (Sugano et al., 1992).

Areas of incomplete demyelination were first identified by Marburg in 1906 (Marburg, 1906). The term *Markschattenherde*, translated as shadow plaque, was introduced by Schlesinger 3 years later (Schlesinger, 1909). These were some of the first pieces of evidence hinting at the capacity for repair of damaged myelin in MS – further studies later provided strong evidence that they represent completed remyelination of a previously demyelinated plaque (Lassmann, 1983). Other types of lesion include the previously discussed Balo's concentric plaque and so-termed preactive lesions – areas in which there is evidence of inflammation, limited breakdown of the blood-brain barrier and reduced myelin density (De Groot et al., 2001; Gay et al., 1997).

Relatively recent advances in our understanding of the pathogenesis of tissue injury in MS have revealed a degree of heterogeneity not previously appreciated. In 2000, Luchinetti and colleagues demonstrated 4 distinct patterns of tissue damage in a biopsy and autopsy series of 84 patients with MS (Lucchinetti et al., 2000), distinguished by a combination of their cellular reaction, patterns of oligodendrocyte loss and the immunopathological picture.

In the first, termed basic general pattern, there is a dominant class 1 MHC-restricted T-lymphocyte and macrophage infiltrate with activation of resident microglia. Macrophages produce a variety of oxidative and nitrative molecules (Witherick et al., 2010), cytotoxic cytokines (Cannella and Raine, 1995) and proteolytic and lipolytic enzymes (Cuzner et al., 1996) which further mediate tissue damage. The macrophages and microglia form clusters that are closely associated with

demyelinating fibres and disintegrating axons, but are also dispersed throughout normal appearing white matter. Class 1 MHC molecules are found on various glial cells including oligodendrocytes (Höftberger et al., 2004) with $CD8^+$ T-cells in close association (Neumann et al., 2002) and with features suggestive of a cytotoxic interaction resulting in characteristic apoptosis. Areas of tissue injury form confluent demyelinated lesions with axonal sparing. New oligodendrocytes are recruited to the area of damage and remylelination is extensive and frequent. In more advanced cases with a progressive phenotype, oligodendrocyte numbers dwindle and remyelination is infrequent (Prineas et al., 2001).

In pattern II, there is antibody-mediated demyelination in addition to the mechanisms found in the basic general pattern of lesion. Deposition of antibodies is accompanied by activation of the complement cascade and absence of complement inhibitory proteins (Storch et al., 1998). Complement activation is only observed in the earliest stages of lesion formation. The antibody-mediated demyelination is associated with greater numbers of B-cells than pattern I, and resulting plaques are more sharply demarcated. Oligodendrocyte recruitment and evidence of extensive remyelination is prevalent in pattern II lesions which are effectively indicative of early chronic disease. Neuromyelitis optica represents an archetypal example of antibody mediated demyelination.

In pattern III there is augmentation of tissue destruction by hypoxic mechanisms thought to include oedema and inflammation of vessel walls and impairment of mitochondrial function by reactive oxygen and nitrogen species. Lesions contain

fewer foamy macrophages and microglial activation is less marked. Chemokine expression is more in keeping with an ischaemic lesion rather than the pattern seen in the more 'classical' MS patterns – I and II. Balo's concentric sclerosis is one of the more extreme examples of pattern III pathology. Elsewhere, plaques are ill-defined and there is characteristic preservation of myelin immediately surrounding the small vessels on which the lesions are centred (Aboul-Enein et al., 2003).

In pattern IV there is augmentation of demyelination that is thought to be related to a genetically-determined heightened susceptibility. Such lesions are seen in patients with functional deficits in ciliary neurotrophic factor and more aggressive clinical disease (Giess et al., 2002). Candidate genes thought to alter outcomes in inflammatory demyelinating disease include apolipoprotein E (Fazekas et al., 2000), mitochondrial DNA (Mojon et al., 1999), spinocerebellar ataxia gene 2 (Chataway et al., 1999) and p53 (Wosik et al., 2003).

Patterns of demyelination classified according to the above criteria are strikingly homogenous when looking at tissue samples from an individual with MS taken at a single time point (Lucchinetti et al., 2000). Clear differences are seen between individuals however, suggesting that the pattern changes according to disease stage. In the acute phases of the disease pattern III predominates with patterns I and II more commonly encountered in the early chronic phases of the disease. The clinical manifestation of these patterns are most evident in Balo's sclerosis and Devic's disease in which pattern IV and pattern II respectively convey the degree and nature of tissue destruction.

Immunology of Multiple Sclerosis

The establishment of the sclerotic plaque is the culmination of several processes including inflammation, demyelination, remyelination and gliosis. The relationship between these disease processes and the neurodegeneration that is thought to be responsible for the accumulation of disability in the condition is yet to be fully reconciled and remains in some quarters a point of debate. The most prevalent theories hypothesise that the index event is regulatory failure of migrating lymphocytes though it has been argued that immunological activation may be a response to an underlying neurodegenerative process (Stys et al., 2012), a theory not without merit. Whilst the debate persists, it is undeniable that the inflammatory process is central to manifestation of the disease.

The inflammatory model/doctrine states that disease begins with transition from physiological immune surveillance to excessive migration of auto-reactive lymphocytes across the blood-brain barrier and establishment of an immune response within the CNS parenchyma. The disease was traditionally thought to be a T-cell dependent process with macrophage-driven demyelination due to aberrant targeting of a myelin-specific autoantigen. Evidence for the central role of T cells includes the presence of Th (T helper) 1 cytokines, receptors and cells in the CSF, circulation and lesions of MS patients (Cannella and Raine, 1995; Merrill, 1992; Navikas and Link, 1996). It was commonly held that disease progression was driven by $CD4^+$ effector cells. Failure of regulatory lymphocytes to induce apoptosis in these cells has been demonstrated to be due to both afferent (regulatory cells from

patients with MS fail to suppress effector cells in-vitro (Viglietta et al., 2004)) and efferent mechanisms (overexpression of β-arrestin 1, a promoter of $CD4^+$ cell survival, prevents effective apoptosis in the effector cell population (Shi et al., 2007)). Further evidence for the central role of these cells comes from the fact that $CD4^+$ T cells polarized to the Th1 phenotype are thought to play a central role in the animal model of MS, experimental autoimmune encephalomyelitis (Khoruts et al., 1995).

With gathering evidence the story of antigen specifics has become more complicated. Whilst myelin proteins continue to be implicated as a target for immune system activity, other antigens such as αB crystalline (Ousman et al., 2007) and neurofascin (Mathey et al., 2007) have been identified as important in tissue targeting and destruction. In recent years it has also become clear that the immunological interplay is not as simple as first thought. Evidence countering the central role of $CD4^+$ cells and broadening the repertoire of implicated immunological cells includes; MHC class 1-restricted, CD8-positive cells are the predominant cell type found in active MS lesions (Babbe et al., 2000); lymphocytes may not be present in early demyelinated lesions; perivascular inflammatory cuffs can occur in normal appearing white matter (Barnett and Sutton, 2006). In addition, therapies such as anti-interleukin 12p40 that targeted $CD4^+$ T cell function have proved ineffective in clinical trials (Segal et al., 2008). It has also recently been discovered that inflammation in EAE is driven by an alternative T-lymphocyte subtype that secretes interleukin 17, effectively disrupting the blood-brain barrier and allowing neurotoxic Th17 cells direct access to the CNS (Kebir et al., 2007).

These novel insights have prompted further interrogation of the underlying immunology of the condition and redirected focus to alternative cell types that may contribute to the pathogenesis of MS. Previously unknown contributors to the disease process include the Th17 cells (producing IL 17) discussed above, B cells, $CD8^+$ cells and both $CD4^+$ and $CD8^+$ T-regulatory cells (Kasper and Shoemaker, 2010). Other effector populations include $CD56^+$ natural killer cells, invariant NK cells and stem cells (Kasper and Shoemaker, 2010).

The plethora of immune cells involved in the initiation of inflammation accumulate at the site of myelin injury and through expression of pro-inflammatory cytokines recruit resident microglia. These cells are activated, delivering tissue necrosis factor α at the opsonised oligodendrocyte-myelin complex resulting in cell death and myelin destruction (Zajicek et al., 1992). Lesions are associated with axonal injury, even transection and associated Wallerian degeneration, and as the disease becomes progressive there is radial expansion of areas of white matter injury.

Axons receive trophic support from oligodendrocytes. Unsurprisingly, even early in the disease course, axonal damage is detectable in the lesions of MS (Trapp et al., 1998). As well as terminal axonal ovoids (an indicator of axonal transection) in active and other lesions, axonal loss is identified in normal appearing white matter and the unmyelinated cells of the retinal nerve fibre layer. Mechanisms postulated to underlie these findings in apparently 'normal' or unmyelinated tissue includes mitochondrial dysfunction secondary to oxidative and nitrative stress (Bolaños et

al., 1997) - the apparent size-dependent loss of axons in NAWM supports disruption of energy supply as a likely mechanism (Stys, 2005). As outlined elsewhere, it is the cumulative loss of axons, eventually exhausting the CNS functional reserve that underlies progressive disability (Compston and Coles, 2002).

Conventional therapy

We have earlier briefly discussed the natural progression of MS and the transformation from repeated inflammatory-predominant episodes with complete or near-complete recovery of symptoms to one of progressive clinical decline as a result of cumulative axonal damage in an environment of dwindling trophic support. Attempts at disease modification have thus far focussed on one of two approaches to the inflammatory component of the condition; immune stimulation on the basis that either persistent viral infection or immunoregulatory defect was central to disease pathogenesis; and immuno-suppression, initially using agents that were largely non-specific.

Several studies using immunostimulants were pivotal in developing our understanding of disease mechanisms, providing unambiguous evidence that clearly redirected research efforts. Of particular note was a study of interferon-γ (Panitch et al., 1987) which demonstrated excess disease activity following 8 injections over a 4 week period in association with an increase in MHC class II-positive circulating lymphocytes. Efforts were subsequently redirected towards

attempting to ameliorate inflammatory activity in increasingly specific ways. Recommendations from the interferon-γ trial authors included trials of the interferon-γ inhibitors interferon-α and interferon-β, subsequently the bedrock of disease modification for the next decade and more.

Conventional disease-modifying therapy in multiple sclerosis has focused on attempting to suppress or modify the inflammatory component of the disease process. The argument is made that preventing or reducing the frequency of discrete inflammatory events will in theory delay the point at which the patient's burden of disease facilitates entry into the progressive phases of the condition in which inflammation is not prominent and immunotherapies have no theoretical beneficial role to play. Whilst the debate remains keenly contested in certain quarters, these predictions have not been realised in the most comprehensive analyses of disease-modifying drugs that have thus far been performed (Mantia et al., 2013; Shirani et al., 2012; Tur et al., 2011). These results have however informed concepts of the disease with prevalence given to the theory of a threshold of inflammatory-mediated injury beyond which neurodegenerative mechanisms are the primary pathology.

The first licensed treatment for disease modification in MS in the EU was interferon-β 1b, awarded in November 1995 on the basis of a single pivotal study (1993; 1995) which demonstrated a reduction in annual exacerbation rate from 1.27 in the placebo group to 0.84 in the 8-MIU treatment group. The reduction of

inflammatory activity on MRI imaging of the brain heightened the impact of the early study.

The next EU marketing licence was awarded to glatiramer acetate a year later after demonstration of a 29% reduction in annualized relapse rate compared with placebo (Johnson et al., 2001). Similar results were seen in the pivotal trials of interferon-β 1a (Avonex, Rebif) (1998). Both were granted licenses towards the end of the 1990's. The introduction to clinical practice of these medications was somewhat erratic and incoherent due in part to an expectant and hitherto underserved patient population and aggressive marketing and promotion by the drug's manufacturers. Several monitoring schemes were established to try and assess long-term efficacy and in some instances facilitate the use of expensive drugs that were subject to government rationing (such as the UK risk sharing scheme). These have confirmed the initial findings of an approximately 30% reduction in relapse rate and provided further evidence of long-term safety (Ford et al., 2010; Palace et al., 2015). None however have thus far demonstrated any convincing evidence of long-term modification of disease course and disability accrual. The mechanisms of action of beta interferons that are thought relevant in the context of MS include inhibition of T-cell activation, modulation of anti- and pro-inflammatory cytokines and restriction of aberrant T-cell migration – though the precise mechanism is not definitively established.

Alongside glatiramer acetate (which targets T-cell peptide binding) several drugs that target T-cell populations have been trialled with the recent emergence of

monoclonal antibodies that are able to target single components of the immune system. Alemtuzumab was the first humanised chimeric monoclonal antibody to be produced. It targets CD52 which is present on all lymphocytes and some monocytes, subsequently activating complement and mediating cytotoxic mechanisms leading to effective depletion of lymphocyte populations (Xia et al., 1993). Trial datasets have also suggested neuroprotective effects of the medication (Jones et al., 2010). A phase II clinical trial demonstrated significant reduction of relapse risk and sustained accumulation of disability of >70% compared to interferon-β 1a (Coles et al., 2008). Although the 5 year follow up showed maintenance of the reduced risk of sustained disability accumulation (Coles et al., 2012), subsequent phase III studies have not replicated the finding (though this may be explained by atypical performance of the control group (Cohen et al., 2012a)).

Natalizumab is a humanised monoclonal antibody that targets lymphocyte α4β1-integrin, preventing interaction with VCAM-1 on endothelial cells and consequently inhibiting migration across the blood-brain barrier. The pivotal trial of Natalizumab demonstrated 68% reduction in relapse rate compared with placebo with reduction of the risk of sustained progression of disability by 42% over two years (Polman et al., 2006).

Both of the monoclonal antibodies currently licenced for the treatment of MS have had significant issues with adverse side effects; Natalizumab with the development of progressive multifocal leukoencephalopathy (Bloomgren et al., 2012) and Alemtuzumab with the development of a number of autoimmune diseases

including idiopathic thrombocytopenic purpura and Grave's disease (Cossburn et al., 2011). When compared with the interferon's side-effect profile, these potential complications limit use which is restricted to those with more aggressive disease.

In the last few years several oral agents have been licensed for use in RRMS – these are all immuno-modulatory agents with varying degrees of potency and side-effect profiles. The first to be licensed was Fingolimod (a sphingosine 1-phosphate agonist which prevents lymphocyte egress from lymph nodes) which received recommendation as a second-line agent in English clinical guidelines. In pivotal studies relapse rate was reduced by >50% compared with placebo (Kappos et al., 2010). As with the monoclonal antibodies, adverse events were more prominent than seen with older 'injectables' with mortalities in trial participants due to disseminated viral infection (Cohen et al., 2010). More recently, dimethyl fumarate, a fumaric acid ester whose actions include reduction of oxidative stress and alteration of chemokine/cytokine profile (producing an anti-inflammatory effect) has been licensed for use in RRMS. It has been shown to reduce relapse rate by 48-53% compared to placebo (Gold et al., 2012). There have been reported cases of PML in the context of its use (though none during treatment for MS) (Ermis et al., 2013). The third oral medication to receive a license for the treatment of RRMS is Teriflunomide, a selective immunosuppressant that ameliorates lymphocyte proliferation by inhibition of dihydroorotate dehydrogenase, reducing pyrimidine synthesis. The precise mode-of-action of the medication in the context of MS remains to be definitively established. Teriflunomide represents the active element of the prodrug leflunomide which has been used for some time in

rheumatoid arthritis. In RRMS it was shown to reduce annual relapse rate by 31% (O'Connor et al., 2011) and is now licensed as first line therapy.

Inflammation vs neurodegeneration

All licensed disease modifying drugs used in RRMS have shown throughout their development, repeated and in certain circumstances, profound suppression of inflammatory activity when observed in-vivo through the medium of magnetic resonance (MR) imaging. Therapeutics have become increasingly specific in their ability to suppress ever narrower elements of the immune system. Despite this, none of the trials have thus far been able to demonstrate any sustained effect on long-term disability accumulation. This has informed and served to refocus the debate surrounding disease mechanisms and the relationship between inflammation and axonal loss which underlies the development of long-term disability accrual.

There are four theoretically possible explanations for the relationship of inflammation and neurodegeneration; that inflammation is the sole pathological mechanism; that neurodegeneration is the primary pathology with secondary or reactive inflammatory changes as a result; that the two processes both occur completely independently; or that inflammation is the index event with neurodegeneration the result of the attainment of a (pathologically lowered) threshold beyond which axonal integrity cannot be maintained. Evidence from

natural history and therapeutic studies have both served to inform the debate and illuminate the role of axonal loss in the progressive variants or stages of the disease.

Evidence from natural history studies;

This includes work analysing the Rennes MS database that suggested two distinct stages of disability progression within MS (Leray et al., 2010). Using Kurtzke's Expanded Disability Status Scale the study analysed time to reach Disability Status Scale (DSS) 3 (mild-moderate disability but fully ambulant) - phase 1, and time from DSS 3 to DSS 6 (requirement of unilateral assistance to mobilise) - phase 2, in both relapsing remitting and progressive onset disease. The study established that phase 2 duration was almost identical irrespective of the duration of phase 1. Kremenchutzky and colleagues (Kremenchutzky et al., 2006) chose to group patients into those with secondary progressive disease, single relapse at onset or no relapses at onset ie primary progressive disease, and identified a similar speed of progression of disability in all 3 groups. The researchers concluded that the stereotyped nature of the progressive phase suggests common functional differential susceptibility to a neurodegenerative process. Furthermore they highlighted the prevalent distal corticospinal tract dysfunction that underlines much of the disability accrual in progressive disease and hypothesised that this may represent a central dying-back axonopathy which is independent of plaque location or burden. They include in their conclusions the possibility that the neurodegenerative element may in some cases be the primary pathology.

Confavreux and Vukusic used similar natural history data from the Lyon MS cohort to draw slightly different conclusions (Confavreux and Vukusic, 2006a). They focused on age at attainment of disability milestones rather duration of disease and concluded that prognosis in MS, regardless of disease phenotype, is to an extent, age-dependent. They hypothesised that alongside inflammatory and degenerative mechanisms there may be accelerated age-related mechanisms contributing to the disability seen in progressive disease. In more wide-ranging data published contemporaneously (Confavreux and Vukusic, 2006b), the same authors used data from all forms of the disease to draw ambitious conclusions about a unifying concept of the disease which they describe as a 'position of complexity rather than true heterogeneity.' Essentially they believe the different disease phenotypes to be variants of the same disease processes; relapsing–remitting disease can be regarded as multiple sclerosis in which insufficient time has elapsed for the conversion to secondary progression; secondary progressive forms as relapsing–remitting multiple sclerosis that has 'grown older'; and progressive from onset cases as multiple sclerosis 'amputated' from its usual preceding relapsing–remitting phase.

Evidence from therapeutic studies;

The predominant contemporary doctrine regards the inflammatory component of the disease as the index or primary event (though as discussed above this concept is challenged in some quarters). Treatment of the underlying disease process, so-called disease modification, has universally centred on manipulation of the immune system and inflammatory response. Evidence for long-term impact on disability

progression has not yet been produced. Whilst this is in part due to limitations on the duration of therapeutic studies in the context of a chronic disease that has a 30-plus year natural history, this failure does leave room for doubt about the likely avenues of treatment that will hopefully one day result in a cure for the disease.

The disconnect between suppression of inflammation and clinical progression is notably demonstrated by studies of anti-CD52 monoclonal antibody in MS. Early trials of the antibody were performed in patients with secondary progressive disease (Coles et al., 1999); the study demonstrated almost complete cessation (>90%) of MRI evidence of inflammatory activity for 18 months (replicating findings of earlier proof-of-concept studies (Moreau et al., 1994)) in the progressive cohort, together with reduction of relapse rate from 0.7 per patient per year to 0.02 per patient per year at a mean follow up of 6.7 years. Effective suppression of inflammation was again demonstrated (T1 lesion volume and proton density) on follow up scans a further 5.8 years later. Despite the suppression of inflammatory activity, disability continued to increase by 0.2 EDSS points per year with associated brain atrophy attributable to axonal loss demonstrated with MRI spectroscopy. Interestingly, deterioration was correlated with inflammatory disease activity at baseline. Two patients in the study showed continuing cerebral atrophy despite clinical stability. The findings suggested that axonal degeneration is conditioned by prior inflammation and proceeds despite its effective suppression, and that this is responsible for disability accrual. The authors concluded that results supported the hypothesis that immunosuppressive treatment must be given early in the disease course 'before the consequences of inflammation are irretrievably established'. In

subsequent work the same investigators administered Campath-1H (anti-CD52 monoclonal antibody) to 22 patients with early (total of 60 patient years pre-treatment) RRMS (Coles et al., 2006). In those in whom EDSS had improved at 1 year, the changes were sustained at 24 months (mean EDSS -1.4); the treatment had effectively stabilised EDSS. These results are in stark contrast to the findings in SPMS and prompted the authors to speculate that early treatment to stop inflammatory demyelination may prevent long-term axonal degeneration.

Alternative therapeutic strategies

In summary, trial results make it clear that inflammation is key in the establishment of the demyelinated plaque, and appears to condition the axonal loss underpinning progressive disability. In theory, the cessation of inflammation prior to initiation of the cascade of processes that lead to loss of axonal integrity may prevent disability, but the ability of powerful immunosuppression to achieve this remains to be established. It is also important to factor in too, that currently available therapeutics that most effectively terminate inflammatory activity (with Campath-1H) comes at a cost of potentially significant side effects (Cossburn et al., 2011). A number of additional approaches are needed if we are to start to address the as yet unmet therapeutic needs of this cohort of patients and prevent the neurodegeneration that most contributes to the burden of disease. This includes but is not restricted to;

1) Prevention of diffuse microglial activation and on-going insult to the axon-glial unit
2) Axonal protection against
    a. acute injury
    b. chronic degenerative processes
3) Enhancement of repair mechanisms, particularly in the context of myelin
4) Promotion of plasticity
5) Promotion of axonal regeneration

The solution to each of these individual problems is unlikely to have a meaningful impact on progress in the search for a cure (supported by the absence of identifiable single gene defects in aetiological explanations), but in combination or in total, may bring success to swelling research efforts.

Cell therapy for Multiple Sclerosis

The failure of conventional therapy to significantly alter the disease course in Multiple Sclerosis has already led investigators (and charlatans) to explore alternative therapies – some with very little in the way of theoretical or proven in-vitro or in-vivo mechanistic explanation. Among the scientific community, cellular therapies have gained prominence. Whilst the proposed mechanisms of

therapeutic effect have shifted somewhat, translational work is now beginning to explore undoubted pre-clinical promise.

The initial mechanisms by which it was thought exogenous stem cells would confer their treatment effect in MS was through replacement of tissue lost or damaged. This was felt to be particularly pertinent to replacement of myelin and oligodendrocytes (the target of inflammation in MS); in contrast, the replacement of neurons and axons and their innumerable connections would appear to provide an infinitely more complex, possibly even insurmountable problem. These early hopes were driven by several influential papers including the work of Blakemore and colleagues who demonstrated successful remyelination of demyelinated CNS lesions using direct implantation of exogenous sciatic Schwann cells (Blakemore, 1977). With hindsight this approach (particularly in the context of MS) appears somewhat misguided with efforts now beginning to focus on more widespread and varied protective and reparative effects of cellular therapy. We will begin by looking at the initially proposed modes of action, particularly remyelination and the cells involved, thereafter following the evolution of cellular therapy chronologically.

Remyelination

Early work by Bunge and colleagues (BUNGE et al., 1961) provided some of the first detailed studies of the mature mammalian brain's ability to remyelinate following experimental lesioning. The human brain's capacity for remyelination was first

discovered over 40 years ago by Perier and Gregoire who identified glial processes wrapping around axons at the periphery of lesions in multiple sclerosis (MS) post-mortem tissue (Périer and Grégoire, 1965). These findings were supported by the realisation that shadow plaques represent areas of remyelination (Prineas and Connell, 1979) and also by their subsequent electron microscopic evaluation. Although at first this remyelination was thought to be sparse and largely functionally ineffective, subsequent data has shown that remyelination occurs much earlier than first thought (Merkler et al., 2006) and far more extensively - white matter lesions have been shown to have high rates of remyelination with only 5% being completely demyelinated and an average lesion remyelination extent of 47% (Patani et al., 2007). Work by Albert and colleagues showed similar results and went further in demonstrating higher rates of remyelination in cortical lesions (present in 29/30 patients with chronic disease) compared with white matter (evidence of remyelination in more than 2/3 of lesions) (Albert et al., 2007). The origin of new myelin formation has remained obscure though it was recognised early on that, as might be expected, most is achieved through cells of oligodendrocyte lineage.

Cells that have been demonstrated to affect a degree of repair in demyelinated tissue include oligodendrocyte precursor cells and endogenous neural stem cells. Picard-Riera and colleagues used lysolecithin-induced demyelination in a murine model of MS and documented neural progenitors of the sub-ventricular zone migrating to periventricular white matter and giving rise to both astrocytes and oligodendrocytes within lesioned tissue (Picard-Riera et al., 2002).

Oligodendrocyte precursors have been identified in both normal brains and in MS post-mortem tissue (Scolding et al., 1998). These cells have been shown to proliferate (Redwine and Armstrong, 1998) and migrate (Carroll and Jennings, 1994) in response to demyelination. They also show several morphological adaptations (Levine et al., 2001) and changes to patterns of gene expression (Fancy et al., 2004) (which play a crucial role in the embryological differentiation of oligodendrocytes) in response to CNS inflammation.

Further characteristics of endogenous remyelinating cells were elucidated by the work of Snethen et al (Snethen et al., 2008) who examined the expression of the neural stem cell markers Nestin and Mushashi (found on early neural progenitors) in MS post-mortem tissue. Nestin-positive cells were identified in MS lesions in high numbers, and were also identified within normal appearing white matter. Mushashi co-labelling was evident in almost 90% of Nestin-positive cells in lesions, providing further confirmation of the presence of neural precursor cells in lesioned areas of the brain. Furthermore, within lesions a high proportion of astrocytes co-expressed nestin; and nestin-positive cells also expressed oligodendrocyte precursor cell markers, though in small numbers. Evidence was also seen of Nestin-positive cells colabelling with doublecortin, a marker of immature neuronal lineage cells, lending weight to the possibility that a degree of axonal regeneration might also be achieved by endogenous stem cells. Despite the presence of functionally significant numbers of progenitor cells, remyelination is frequently incomplete. Some of the reasons for this functional deficit have been identified (hyaluronan accumulation and expression of PSA-NCAM) and provide further potential

therapeutic targets (Fancy et al., 2010). Additional rationale for the failure of remyelination include ageing of the progenitor cell population, exposure to direct attack by disease mechanisms and the recurrent nature of demyelinating episodes.

Which cells?

One of the first fundamental questions that was posed as the science behind re-growing elements of the injured CNS was approached was of course which cells would be able to perform such feats. Given that most endogenous repair is carried out by oligodendrocyte progenitor cells, they appeared the most logical source of cells for replacement therapy. Although they can be isolated from the adult human brain (Scolding et al., 1995) and proliferate in-vitro, the numbers involved are relatively low (Roy et al., 1999) and they show only limited migration in response to CNS tissue injury (Franklin et al., 1997). Given these logistical challenges, alternative sources of remyelinating cells were considered including but not limited to Schwann cells (as originally used by Blakemore), olfactory glia from the olfactory bulb, xenogenic transplanted cells and human stem cells. Stem cells have many properties that made them clear frontrunners in the search for an effective candidate - the most fundamental of which is their multipotency. Stem cells are attached to basal lamina and are able achieve asymmetric mitosis, generating both progenitor cells and self-renewing. In mature organisms these cells are central to tissue repair and regeneration and as such are particularly resilient in tissue-injurious environments – one remarkable example of this is the isolation of such

cells from post-mortem tissue (Mayer et al., 2005). Other properties of theoretical value include an apparent tropism for areas of tissue injury and their mitotic potential to generate vast numbers of daughter cells whose fate could be directed along desired lineages.

Broadly speaking stem cells can be either embryonic or adult-derived, though the category of induced stem cells has been introduced in the last 10 years. Embryonic stem cells (ESCs) are found in the inner cell mass of blastocysts and were first isolated from mouse embryos in 1981 (Evans and Kaufman, 1981). Human ESCs have also been isolated (Thomson et al., 1998) and differentiated along neuronal lineages (Carpenter et al., 2001). The technology also exists to isolate neural precursor cells directly from embryonic tissue (Reynolds and Weiss, 1996). Whilst benefit has been shown in animal models of other neurological conditions (Studer et al., 1998) the practical challenges in translating their use to in-human therapeutic studies are largely prohibitive (Armstrong et al., 2000). The use of embryonic stem cells comes with two clear problems; the first is the propensity of the cells to form teratomas (Bjorklund et al., 2002) and the second, the ethical implications of harvesting cells from a pre-implantation blastocyst (or in the case of neural precursor cells from embryonic tissue) which has the potential to develop into/already is an individual human being. Translational use of these cells has therefore been limited.

The ethical issues of ESCs (and indeed of therapeutic human cloning) have been somewhat circumvented by the development of techniques to reverse adult cell

differentiation to produce induced pluripotent stem cells (iPSCs). iPSCs were first generated by Takahashi and Yamanaka in 2006 (Takahashi and Yamanaka, 2006); the technique used just four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) to generate pluripotent stem cells from adult mouse fibroblasts. This work was followed by the generation of human iPSCs by both Yamanaka (Takahashi et al., 2007) and a group at the University of Wisconsin-Madison (Yu et al., 2007). iPSCs have almost identical properties to ESCs including their morphology, self-renewal, proliferation, gene expression, and ability to differentiate into all three germ cell layers both in vitro and in the context of teratoma formation. As with ESCs however, safety concerns have limited translational work and their current role is primarily in modelling disease for the purposes of laboratory study. Mouse iPSCs have been used to generate neural precursor cells and OPCs (Czepiel et al., 2011) and have been demonstrated to ameliorate pathological and clinical features of disease in EAE in mice when delivered intrathecally (Laterza et al., 2013). Interestingly the effects were found to be due to neuroprotective effects rather than direct integration and tissue repair. A degree of the neuroprotective effect was conferred through leukaemia inhibitory factor (LIF) which promotes endogenous remyelination. Human iPSC-derived OPCs were shown by Wang and colleagues to myelinate a myelin basic protein-deficient *shiverer* mouse model when implanted neonatally (Wang et al., 2013). The efficiency of myelination seen with the iPSC-dervied OPCs was higher for that seen with ESC-derived progenitors. Transplantation of allogenic OPCs from whichever source is fraught with problems related to compatibility and rejection, a particular concern in the context of the aberrant immune environment encountered in MS (Keyoung and Goldman, 2007).

Additional practical difficulties arise from the problem of 'epigenetic signature', a resident epigenetic profile that encourages iPSCs to differentiate down their original lineage (Bar-Nur et al., 2011). Whilst iPSCs have the distinct advantage of being genetically identical to the donor individual, the functionality of OPCs derived from iPSCs in individuals with MS or other disease remains to be determined.

The other principal category of stem cells are those derived from a mature (usually mammalian in the context of biomedical research) organism – termed adult or somatic stem cells. These are linage-restricted cells resident in multiple tissues in the adult organism, able to effect repair and regeneration in response to natural cell turnover or cell loss in the context of trauma or disease. The presence of such cells in humans, especially in tissues with a high turnover such as skin or blood was first predicted at the beginning of the 20th century by the Russian Histologist Alexander Maximow who developed the unitarian theory of haematopoiesis. Adult stem cells are numerically infrequent cells that reside within tissues within the mature, adult organism. Whilst showing multipotency, and able to replicate asymmetrically to both self-renew and produce fate-restricted progenitors with more limited proliferative capacity, these cells are thought to be restricted to the lineage of their organ of origin. Haematopoietic stem cells have been the most widely studied of human somatic stem cells, with over 50 years of detailed laboratory characterisation and substantial safety data acquired through what has become routine clinical use, primarily in the treatment of malignant haematological disease. Adult stem cells are found in tissues throughout the body including in the brain (Arsenijevic et al., 2001; Weiss et al., 1996). From the perspective of tissue

replacement, hese cells have also successfully been used to generate oligodendrocyte precursors, a prerequisite for effective remyelinating potential (Zhang et al., 2000).

Bone marrow-derived Adult Stem Cells

Bone marrow contains a readily accessible supply of a number of adult stem cell populations that are increasingly being exploited in wide-ranging medical conditions including myocardial infarction (Núñez García et al., 2015), liver cirrhosis (Margini et al., 2014), osteoarthritis (Marmotti et al., 2014), several respiratory diseases including pulmonary hypertension, COPD and asthma (Jones and Rankin, 2011) and degenerative eye disease (Mead et al., 2015); indeed bone marrow-derived stem cells are currently being explored as potential therapy in diseases affecting the majority of the human body's organs. The so-called bone marrow stem cell compartment consists of a number of cell populations, traditionally thought to include haematopoietic stem cells, mesenchymal or stromal cells and endothelial progenitor cells. As our understanding develops and more cell types are characterised it is becoming apparent that differences between cell subsets are much more nuanced than previously thought with the suggestion that several populations that have been described in the literature with varied isolation techniques do in fact represent very similar or even overlapping sub-populations. These include multipotent adult progenitor cells (MAPCs) (Serafini et al., 2007), marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito et al., 2004)

and multilineage-differentiating stress-enduring (MUSE) cells (Wakao et al., 2011). Very small embryonic-like stem cells (VSELs) are another cell type that can be isolated from the bone marrow niche and are argued by some to account for much of the multipotency attributed to various bone marrow-derived cell subsets (Ratajczak et al., 2014). VSELs can be isolated by flow cytometry using a series of cell markers (Sca-1^+; $CD45^-$; lin^-) and have many properties in common with pluripotent embryonic stem cells including open-type euchromatin DNA, a large nuclear: cytoplasmic ratio and expression of several embryonic lineage markers including stage-specific embryonic antigen 1 (SSEA-1), Oct-4, Nanog, and RNA exonuclease 1 homolog (Rex1) (Kucia et al., 2005). These and other discoveries have prompted efforts to start to try to reclassify and categorise populations of stem cells residing in the bone marrow stem cell compartment (Zhang and Huang, 2012). Broadly speaking the cells can be classified as haematopoietic or non-haematopoietic stem cells with further delineation of the non-haematopoietic population yet to gain consensus.

Haematopoietic stem cells;

The restoration of haematopoiesis following myeloablative therapy by haematopoietic stem cells is the most obvious therapeutic example of the ability of somatic stem cells to restore function through replacement of tissues lost or damaged and is in routine clinical use in haematology clinics around the world. Haematopoietic stem cells were traditionally thought to express CD34, CD45 and Thy-1 with absence of multiple markers of lineage commitment (lin^-). It has become apparent with time, however, that HSCs are a much more heterogeneous

population than first thought (Eaves, 2015). Current isolation techniques including using physical and biochemical (enzymatic) properties along with patterns of surface markers (specifically signalling lymphocyte activation molecules or SLAM markers) gives rise to demonstrably different types of long-term repopulating cell phenotypes with distinct repopulating kinetics (Eaves, 2015). Clinical use of HSCs for the purposes of reconstitution of the haematopoietic system does not require separation of these cell subsets which are collected directly from red bone marrow or from G-CSF-mobilised peripheral blood.

Endothelial progenitor cells;

Endothelial progenitor cells are a population of cells that have the capacity to migrate to the peripheral circulation and differentiate to form mature endothelial cells. They express a number of cell markers including CD34, CD133, and vascular endothelial growth factor (VEGF)-receptor 2. Maturation is associated with loss of CD133 expression and expression of CD31, vascular endothelial cadherin and von Willebrand factor (Hristov et al., 2003; Hristov and Weber, 2004). Their role in angiogenesis has made them attractive candidates for use in potential therapies for a number of ischaemic conditions including but not restricted to myocardial infarction (Henning, 2011), enhancement of vascular graft endothelialisation (Goh et al., 2015), limb ischaemia (Tateishi-Yuyama et al., 2002) and stroke (Zhao et al., 2013).

Mesenchymal stromal cells;

Mulitpotent mesenchymal stromal cells (variously referred to as mesenchymal stem cells and marrow stromal cells) were first isolated by Friedenstein and colleagues in the 1960s when osteogenic progenitor cells with fibroblast-like morphology were isolated from the bone marrow of adult rats (Friedenstein et al., 1966). The group proceeded to characterise in more detail what were termed colony-forming unit-fibroblasts but it wasn't until 1991 that the term Mesenchymal Stem Cell appeared in the literature. The varying nomenclature is something of a misnomer; the cells are neither mesenchymal (they are not thought to generate haematopoietic cells under normal *in-vivo* conditions), nor are they purely stromal (connective role supporting active cells within the residing tissue). The multitude of properties that these cells have been found to possess, with particular emphasis on tissue repair (suggested by some to be their primary function) are discussed in more detail below. These spindle–shaped cells make up approximately 0.01-0.001% of nucleated bone marrow cells (Xiao et al., 2015) and can be manipulated to differentiate into cells of adipogenic, osteogenic and chondrogenic lineage (Pittenger et al., 1999). The difficulty associated with varying terminology and isolation methods being reported in the literature led the International Society for Cellular Therapy Mesenchymal and Stem Cell Committee to first clarify the nomenclature (Horwitz et al., 2005) and subsequently to establish minimum criteria for the isolation and identification of MSCs, enabling standardisation of a fragmented field (Dominici et al., 2006). The criteria consisted of;

1) Isolation by plastic-adherence under standard culture conditions

2) Expression of specific cell surface markers: express (≥90%) CD105, CD73 and CD90: negative (≤2%) for CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR
3) Ability to differentiate into osteoblasts, adipocytes and chondroblasts in vitro.

Following initial identification in bone marrow, MSCs were subsequently discovered in many other tissues; notably adipose tissue (Zuk et al., 2001) and dental pulp (Bojic et al., 2014) – indeed it is becoming increasingly accepted that MSCs reside in most if not all tissues in the body (da Silva Meirelles et al., 2006).

Interest in MSCs in the field of MS and other neurodegenerative diseases was first piqued at the turn of the century when it was realised that the cells could be manipulated to differentiate into cells of neuroectodermal lineage with obvious implications for the therapeutic strategy of tissue replacement (Sanchez-Ramos et al., 2000). The identification of rapid and widespread transdifferentiation and integration of donor-derived bone marrow cells in the brains of mice lent further support to the potential of the science. Cells were detectable in the brain of the recipient animal after just 3 days with cells eventually detectable throughout the brain including the cortex, hippocampus, thalamus, brain stem, and cerebellum (Eglitis and Mezey, 1997). Cells of bone marrow origin were quite logically then hypothesised to contribute to spontaneous tissue repair in the central nervous system with obvious implications for a number of conditions (Kørbling and Estrov, 2003). Further evidence of a potential physiological role in neural tissue repair came from studies of post-mortem brain tissue from patients given sex-

mismatched bone marrow transplants, where 'transgender' astrocytes and neurons constituted 1% of hippocampal neurons/astrocytes (Cogle et al., 2004).

Alternative mechanisms of MSC activity

Through careful and repeated study of murine models of MS (principally extrinsic allergic encephalomyelitis) and its treatment with MSCs it has become apparent that the cells provide neuroprotection and can ameliorate disease without significant neural differentiation (Gordon et al., 2008), with focus therefore shifting to alternative mechanisms of therapeutic effect.

Cell replacement;

It is still possible that the cells do achieve a degree of cellular replacement but the extent to which this is functionally relevant remains to be established. Whilst the significance of processes including transdifferentation, transdetermination, and dedifferentiation have not been advanced, there is emerging evidence of fusion as a potential mode of tissue repair both in neural tissue (purkinje cells of the cerebellum) and others including cardiomyocytes and hepatocytes (Alvarez-Dolado et al., 2003). Fusion of human MSCs with host rodent neural cells has been documented in-vivo (Kemp et al., 2011), with evidence that other marrow-derived cells fuse in response to inflammation and immune-system activation, clearly relevant in the MS CNS environment (Johansson et al., 2008). This study also identified cell fusion occurring at much higher frequency than previously thought

(10-100 fold) and whilst the functional significance of such fusion remains unanswered in the context of CNS disease, cell fusion has been demonstrated to restore liver function in mice with lethal recessive liver disease (Vassilopoulos et al., 2003).

Heterokaryon formation has been identified in the brains of patients with MS (Kemp et al., 2012) - binucleate heterokaryons were identified at higher frequency in the cerebellum of 6 patients with MS compared with controls. Further mechanism of potential therapeutic benefit in the context of cellular fusion include the discovery that MSCs are able to temporarily fuse with and transfer mitochondria to murine alveolar epithelia in the context of acute lung injury, with functional significance evident through increases in levels of intracellular ATP (Islam et al., 2012). The precise role of cellular fusion remains to be fully established; it could represent a mechanism by which healthy nuclei or genes are delivered to damaged or ageing cells, effectively protecting against degeneration, and circumventing the complex issue of injured neuronal cell (and all of its myriad synapses) replacement.

Immunomodulation;

Immune system modulation with suppression of destructive inflammatory activity and enhancement of reparative elements would have obvious implications in the treatment of MS. MSCs have been shown to have effects on both the innate and adaptive immune systems, inducing profound inhibitory effects on a number of cellular components of the immune system including T cells, B cells, dendritic cells

and natural killer cells (Uccelli et al., 2007) and have been utilised in a number of autoimmune conditions including graft versus host disease and inflammatory bowel disease (Bernardo and Fibbe, 2012). Significant abrogation of disease severity in animal models of MS has been achieved using human MSCs (Gordon et al., 2008) and translational studies are in progress. It should be noted that in the study by Gordon et al the beneficial effects of MSCs were seen without significant CNS infiltration or indeed integration. Inflammation contributes to axonal loss and its abrogation by MSCs has been demonstrated in the animal model EAE to preserve axonal tissue (Zhang et al., 2006). Whilst immunosuppressive effects of MSCs would clearly be desirable and would in theory contribute to therapeutic activity, it is not thought to be their principal mode of action and is likely to be of a much lesser order than levels achieved using powerful contemporary agents that target single cell markers (such as alemtuzumab) or even smaller but eloquent molecular components of the immune system such as NLRP3 (The NOD-like receptor (NLR) family, pyrin domain-containing protein 3) (Coll et al., 2015).

Neuroprotection;

With the accumulation of research data, it has become clearer that the probable principal mechanism of functional benefit of bone marrow-derived stem cells is paracrine activity which provides powerful neuroprotective effects. This is achieved via a number of mechanisms including anti-oxidant activity, provision of trophic support and interaction with resident stem cells.

Amelioration of oxidative/nitrative stress;

Reactive oxygen and nitrogen species are generated as part of cells' normal physiology. Overproduction of ROS or a failure of antioxidant mechanisms can lead to damage to lipids, proteins and nucleic acids in cells and mitochondria, potentially resulting in cell death. There is a constant exposure to low levels of these oxidative/nitrative species in the normal CNS at levels which can easily be dealt with by inherent repair and protection mechanisms. In the inflammatory state these mechanisms can become overwhelmed leading to oxidative/nitrative stress and damage to the basic structural and functional elements of the cells. Reactive oxygen and nitrogen species include superoxide ions, hydrogen peroxide, nitric oxide and peroxynitrite, all of which are produced as part of the inflammatory response. High levels of NO, peroxynitrite and superoxide have been demonstrated in spinal fluid derived from patients with multiple sclerosis (Calabrese et al., 2002) and have a clear potential role in the disease processes. Inflammatory nitric oxide synthase (iNOS) mRNA (iNOS is responsible for the production of NO in the CNS) has been identified in MS plaques (Bö et al., 1994). Evidence for reactive oxygen species involvement comes from both direct and indirect observations - high levels of ROS have been identified in macrophages and microglial cells in mice with EAE (Ruuls et al., 1995), with evidence of lipid peroxidation in post-mortem human tissue (Newcombe et al., 1994) - thought to play a crucial role in early plaque development. Human MSCs have been demonstrated to possess a number of antioxidant properties which help protect against free-radical induced injury including secretion of anti-oxidant molecules, specifically superoxide dismutase 3 (SOD3), with protection of cultured murine cerebellar neurones demonstrated in

vitro (Kemp et al., 2010). MSCs have also been shown to reduce expression of iNOS in microglia, further enhancing antioxidant effects (Kim et al., 2009).

Trophic support

Bone marrow-derived cells have been known for some time to secrete a plethora of cytokines including hepatocyte and nerve growth factors and brain-derived and glial cell line-derived neurotrophic factors, important in the growth, development and maintenance of the human CNS (Crigler et al., 2006). Human MSCs in coculture with mouse neonatal brain cortex express neurotrophin low-affinity (p75) and high-affinity (trkC) receptors and secrete nerve growth factor and neurotrophin-3 (Pisati et al., 2007), clearly demonstrating the functional ability to provide trophic support in the CNS environment, specifically those relevant to oligodendrocytes. Evidence for neuroprotective activity beyond immunosuppression comes from studies demonstrating therapeutic benefit of MSCs in non-inflammatory CNS pathology (Chen et al., 2001). Our group have previously demonstrated that brain-derived neurotrophic factor is secreted by MSCs and ameliorates cell injury in cultured rodent cortical neurons via activation of AKT pathways with a central role of the phosphatidylinositol 3-kinase and MAPK pathways (Wilkins et al., 2009). It has also been demonstrated that MSCs secrete additional neurotrophic factors when exposed to medium conditioned by rodent brain that had undergone experimental traumatic injury (Chen et al., 2002). Modulation of secreted proteins including cytokines and neurotrophic factors are thought to enable redirection of cells from apoptotic to survival pathways (Kaplan and Miller, 2000) and is likely to represent a key therapeutic activity of MSCs.

Interaction with resident stem cells

The unselected bone marrow-derived mononuclear cell fraction (Sasaki et al., 2001) and isolated stromal cells (Akiyama et al., 2002) have been shown to enhance remyelination and repair following intravenous administration in non-immune rodent models of demyelination. The mechanism of action in these and other examples remains obscure. In both studies the cells were genetically labelled and observed to directly myelinate axons in the damaged spinal cord. However, consensus now suggests that direct replacement is likely to be a rare event (Freedman et al., 2010) and consequently interaction with resident stem cells is more likely to be of greater therapeutic value. Evidence for interaction with resident stem cells comes from research showing that human MSCs could significantly increase proliferation of endogenous neural stem cells expressing the stem cell marker Sox2 (Munoz et al., 2005), and the increase in oligodendrocyte lineage cells in lesions following injection of human MSCs into mice with EAE with evidence that this may be a result of MSC-induced changes in neural cell fate (Bai et al., 2009). Bai and colleagues later identified hepataocyte growth factor (HGF) as a key mediator of functional benefit in the administration of MSCs in mouse MOG35–55-induced experimental autoimmune encephalomyelitis. Support for a role for paracrine interaction with resident stem cells was achieved with the demonstration that remyelination in lysolecithin-induced rat dorsal spinal cord lesions could be accelerated by administration of HGF alone (Bai et al., 2012).

Translational studies of human bone marrow-derived cells in MS

Despite the developing consensus regarding the inability of bone marrow cells to effect direct cellular replacement, paracrine reparative activities, neuro-protection, anti-oxidant and immune effects, and interactions with resident neural stem cells are considered to represent valuable potential therapeutic effects of bone marrow-derived cells in neurodegenerative disease. The age of translation of the science to the clinic has begun and several phase I and II studies have been published (Connick et al., 2012; Rice et al., 2010; Yamout et al., 2010). These studies were not designed to detect treatment effects but secondary outcomes did suggest several beneficial effects and phase II randomised and controlled studies are underway.

Much work has been done within the field of neuroscience to elucidate the properties of bone marrow-derived stem cells given the early evidence of migration into areas of CNS disease or damage (Kørbling and Estrov, 2003). Multipotent mesenchymal stromal cells (MSC) have been extensively studied, due in part to their ease of isolation and culture, and has displayed pathophysiological effects that are theoretically beneficial in MS, as discussed above. MSCs have consequently been adopted as the sole cell to be used by some groups interested in cell therapy for neurological disease. A second, simpler, less expensive approach is to use the whole of the bone marrow mononuclear fraction, containing all of the many stem cell sub-populations present in the bone marrow. This approach exploits any properties/cell-cell interactions that these additional cell types possess/participate in and will also include those cells responsible for the neural integration and

transdifferentiation identified in sex-mismatched donor recipients (Mezey et al., 2003). Our group published in 2010 a phase 1 translational study using systemically administered autologous whole bone marrow (Rice et al., 2010). Although primarily powered to analyse safety, a beneficial effect in progressive MS was suggested by neurophysiological outcome measures and a phase II study is now under way. Although much is now known about the biology of the MSC sub-population, the use of whole bone marrow inevitably raises questions about the biological effects of cells other than MSCs that are introduced into the systemic circulation during whole bone marrow mononuclear fraction transfer, as well as their eventual fate.

Additional cells within the bone marrow niche

As discussed above, the make-up of the non-haematopoietic stem cell component of bone marrow continues to be the subject of much debate and attempts at classification are complicated by the wide heterogeneity and dynamic nature of the cell populations. Even within the HSC compartment, new cell subsets continue to be identified and characterised. One of these, $CD133^+$ HSCs, are one of the most promising candidates for a potential role in neural protection. CD133 or prominin-1 was the first member of the prominin family of pentaspan membrane proteins to be identified (Miraglia et al., 1997; Weigmann et al., 1997; Yin et al., 1997). It was recognised in this work to be a marker of primitive $CD34^+$ haematopoietic stem cells capable of long-term repopulation in xenografted animals, and subsequently to

have high clonogenic potential (de Wynter et al., 1998) and significant regenerative capacity (Torrente et al., 2004). Differentiation so far achieved in-vitro by various groups includes cells of both mesodermal (Bonanno et al., 2007) and ectodermal (Bussolati et al., 2005) lineage. $CD133^+$ cells isolated from human neural tissue were demonstrated to engraft, proliferate, migrate and differentiate in the brains of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Uchida et al., 2000). $CD133^+$ cells isolated from the bone marrow compartment have been demonstrated to provide protection to neural progenitor cells in the context of growth factor/nutrient withdrawal - $CD133^+$ cell-conditioned media ameliorated cortical infarct volume in an ischaemic model of neural injury in mice (Bakondi et al., 2009). Further work by this group identified stromal-derived factor 1 alpha (SDF-1) as a key mediator of neuroprotective activity (Bakondi et al., 2011). CD133 is also expressed on endothelial progenitor cells which play a key role in angiogenesis both in wound healing and tumour formation (Ribatti, 2004). The early demonstration that cord-derived $CD133^+$ cells can undergo differentiation into endothelial and cardiomyocytes in-vitro (Bonanno et al., 2007) has led to extensive interest in its potential relevance to cardiac disease, particularly myocardial infarction (Acosta et al., 2013).

Other cell markers of promising significance include Stro-1, a marker of early stromal cell precursors with enhanced proliferative capacity (Simmons and Torok-Storb, 1991), and CD271 low affinity nerve growth factor receptor. CD271 or p75 neurotrophin receptor is a member of the tumour necrosis factor superfamily and has been identified as a marker of primitive mesenchymal cells with greater

clonogenic and differentiative capacity than MSCs isolated by plastic adherence (Quirici et al., 2002). Bone marrow-derived $CD271^+$ cells have also been shown to possess neuroprotective properties, again within the context of growth factor and nutrient withdrawal (Bakondi et al., 2009), and immunosuppressive properties, clearly of potential relevance in the context of MS (Kuçi et al., 2010).

Assessing the impact of therapies for progressive disability

History of disease measures in MS;

One of the main hurdles facing any therapeutic trial aiming to reduce the progression of disability in MS is that of measuring and assessing disability. Current disability outcome measures have significant problems including insufficient sensitivity and reproducibility, inadequate validation and lack of patient subjective components. Despite these problems, a lack of clear alternatives has led to widespread use in therapeutic studies.

It was recognised early in the therapeutic age of MS that further development of disability measures was necessary. In 1995 the National MS Society of the USA convened an international meeting specifically to look at outcome measures in MS clinical trials, concluding that the Expanded Disability Status Scale (EDSS – discussed in more detail below) was neither responsive nor sensitive enough and appointed task forces to look at specific areas for development in more detail

(Whitaker et al., 1995). Despite no little endeavour over the subsequent 20 years, meaningful progress in this area remains elusive.

Clinical parameters

The Expanded Disability Status Scale;

The first attempts to systematise objective clinical rating systems for Neurological impairment in MS appeared in the middle of the 20th century and were quickly developed and refined. In common with many diseases, the emergence of potential therapeutics drove the need for an accurate way of measuring the disease. John Kurtzke worked on one such study (of isoniazid in MS) and through his work developed the Disability Status Scale (DSS) (KURTZKE, 1961), later refined to the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983), still the 'gold standard' clinical parameter to this day. The EDSS is a 10-point ordinal scale containing 20 different levels of disability ranging from 0 (normal neurological examination) to 10 (death due to MS). The score is based on a synthesis of neurological examination findings, ambulation capacity (including requirement of unilateral, bilateral or wheeled aid) and level of independence in undertaking activities of daily living. The scale encompasses a wide range of neurological functions that are relevant to MS including bowel, bladder and cerebral (fatigue, depression and mentation) function.

Although rightly recognised for its remarkable contribution to the study and treatment of MS, the EDSS has widely acknowledged shortcomings (Hobart et al., 2000). The original DSS was criticised for being insufficiently sensitive and unresponsive to clinical change, perceived shortcomings that were addressed in the design of the more detailed EDSS; whilst this improved sensitivity to small changes in Neurological function, it came at the expense of reliability and reproducibility. Further concerns arise from the fact that the scale is heavily weighted towards ambulation and the integrity of the corticospinal motor tracts, and pays little attention to cognitive function. The EDSS is non-linear; the implication of a 1.0 score change has vastly different meaning depending on the patient's position on the scale, and progression is partly therefore a function of baseline score (Weinshenker et al., 1991), limiting potential statistical interpretation and compromising function in therapeutic studies. Administration of the EDSS brings further concerns; it is largely reliant (at least within the lower reaches of the scale) on the neurological examination which is inherently subjective, administration is complex and time-consuming and scoring somewhat ambiguous with poor intra- and inter-rater reproducibility a somewhat predictable consequence (Amato et al., 1988). This variability in the EDSS is particularly troublesome in control cohorts where it undermines the ability to detect treatment effects (thus far prevention of disease progression) in therapeutic studies - Ebers and colleagues used data from the placebo arms of 31 clinical trials and found that disability progression was no more likely in this group than improvement, concluding that existing definitions of disease progression reflect, among other things, measurement error (Ebers et al., 2008). They wrote that surrogates of unremitting disability (utilising the EDSS) in

RRMS ‘cannot be validated’, effectively questioning its role in future therapeutic studies.

Despite these well-recognised concerns the EDSS continues to be used as a primary outcome measure in pivotal trials of therapeutic agents. Its primary advantage is its established acceptance to regulators and extensive historical use enabling comparison between temporally-dispersed cohorts. Several improvements and modifications have been enabled or recommended by the International Advisory Committee on Clinical Trials in Multiple Sclerosis to help improve reliability, reproducibility and ease of administration (Cohen et al., 2012b).

The Multiple Sclerosis Functional Composite;

In 1999 a task force instructed by the National Multiple Sclerosis Society's (NMSS) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis reported on the development of a clinical measurement tool designed to reconcile some of the shortcomings of the EDSS and other clinical rating scales (Cutter et al., 1999). The Multiple Sclerosis Functional Composite (MSFC) comprises of 3 tests designed to quantify upper limb function, lower limb function/mobility and cognitive function with the 9-hole peg test, a timed 25 feet walk and the paced serial addition test. Each component is normalised by conversion to a z-score measured either against a reference control population or the performance of the study population at baseline. Averaging of these 3 components produces a quantitative score on a

continuous scale which is much more amenable to statistical analysis and interpretation. Administration of the test takes just 15-20 minutes, depending largely on the patient and speed of task completion, and it can be done by a trained technician rather than qualified medical (and ideally Neurological) personnel.

Despite the demonstration of correlation with many parameters of MS including clinical (Rudick et al., 2009), imaging (Kalkers et al., 2001) and patient-reported outcomes (Miller et al., 2000), the MSFC has not been acceptable to regulators as a primary endpoint in therapeutic studies. This is primarily because the composite score is abstract, dimensionless and infrequently encountered by physicians, limiting clinical interpretation. Another concern is that using internal baseline data to generate z-scores (as opposed to the reference MS population used by the NMSS task force in designing the MSFC scoring manual), limits capacity for meta-analysis of trial results.

Other methods of quantifying the disease impact of MS currently being explored include composite endpoints such as disease activity free status (clinical and radiological), global measures of activities of daily living (Rankin scale, Barthel index), patient reported outcomes and other novel techniques such as kinematic recording. None of these has thus far been validated.

Magnetic Resonance Imaging

Early use of MR imaging in MS revealed the extraordinary ability of the technology to visualise some of the core pathological features of the condition *in-vivo* (Young et al., 1981). The identification and quantification of subclinical disease activity clearly had significant implications for diagnosis, becoming a key component of diagnostic criteria, as well as prognosis both over the immediate and longer term (Fisniku et al., 2008). Alongside clinically confirmed relapses, imaging parameters were widely adopted as surrogate markers used to demonstrate immunosuppressive effects of novel therapeutic agents in 'proof of concept' studies. These consisted of primarily lesion-based metrics including T2 lesion load and activity, and GAD-enhanced T1 lesion number and volume, both widely employed in phase I and II clinical trials, a crucial step in bringing today's therapeutics into clinical use (Barkhof et al., 2012).

Biomarkers are an attractive alternative to clinical parameters if they are quantitative, reproducible and sensitive to small subclinical changes in disease status. MRI has clearly been a front-runner in this domain for some time; through revelation of the effective suppression of immune activity it has been validated as a trial-level surrogate endpoint for relapse (the clinical correlate of a focus of new inflammatory activity in the CNS) in RRMS (Sormani et al., 2009). The contribution of changes in lesional MRI parameters to variations in disability is however more of a contentious issue; studies have drawn conclusions ranging from a significant to

little/no contributory effect. Ebers and colleagues used clinical data from 31 trial placebo arms to model the relationship of changes in MRI T2 lesion load to both in-trial relapses and disability accumulation (as measured using the EDSS) (Daumer et al., 2009). In the RRMS cohort inclusion of Gadolinium-enhancing lesions in multivariate models made no independent contribution to relapse occurrence and T2 lesion load made no independent contribution to predicting disability. In the SPMS patients included in the analysis, T2 lesion load accounted for less than 5% of variance in disability at the conclusion of the trials. This is contradicted by work by Sormani and colleagues which reports that at a study population level, MRI activity correlates with relapses, and both MRI activity and relapses correlate with end-of-trial disability (Sormani et al., 2011a). Furthermore, in contemporaneously published work the same group reported that a combination of MRI metrics (T2 lesion frequency) and relapse account for up to 100% of the treatment effect on worsening of EDSS at 2 years (Sormani et al., 2011b). Although detailed critique has been made of both findings, particularly that of the positive effect (Wolinsky and Beck, 2011), it is the authors opinion that the emerging evidence of a neurodegenerative component that underlies disability accumulation (and effectively progresses independently of neuroinflammation) provides an altogether more convincing rationale.

The perceived lack of correlation between inflammation in the substrate of the brain and accumulated disability has led to a broad search for alternative biomarkers that seek to quantify the neurodegenerative element of the condition

and allow prediction of disability accumulation. In the context of imaging, these have included 'conventional' MRI features such as T1 hypointense lesions (or persistent black holes), global or component brain atrophy and spinal cord atrophy (as a surrogate for axonal loss) as well as novel sequences and parameters including magnetisation transfer and diffusion tensor imaging. Composite MRI endpoints have been utilised (Wolinsky et al., 2000) but are susceptible to similar criticisms that are made of the dimensionless MSFC, particularly as a trial primary end-point. Other surrogates gaining clinical traction include neurophysiological parameters, serological biomarkers, optical coherence tomography and kinematics.

In seeking to measure the degenerative component of MS, it would seem intuitive to perform pilot work in the progressive forms of the condition and specifically the primary progressive cohort. The heterogeneity of the condition of MS remains to be fully resolved. Evidence to suggest a unifying underlying process particularly in the context of the progressive phases of the condition has previously been discussed. Compared with secondary progression, primary progressive disease is (largely) unencumbered by superimposed inflammatory activity making interpretation and analysis of its measurement more robust. To some it is the ideal substrate for advancing research into disease quantification.

Neurophysiological surrogates of disease

The use of evoked potentials to interrogate the functional integrity of the human CNS was first explored by Dawson in the middle of the twentieth century (Dawson, 1947) and first applied to MS in the 1960's (Baker et al., 1968; Namerow, 1968a, b). Evoked potentials in which sensory nerve fibres are stimulated and the corresponding electrical activity or potential is recorded in the CNS, were initially widely used in the identification of clinically silent disease activity, aiding diagnosis of the condition. The first to be explored in the context of MS was the visual-evoked potential (VEPs) (Halliday et al., 1972), followed by somatosensory (SSEPs) (Small et al., 1978), brainstem auditory (BSAEPs) (Robinson and Rudge, 1977) and electrical (Cowan et al., 1984) and then magnetic (Hess et al., 1986) stimulation-induced motor evoked potentials (MEPs).

Pattern reversal visual evoked potentials revealed an increase in latency in 90% of clinically-confirmed cases of MS (Halliday et al., 1972) and quickly entered clinical use (Halliday et al., 1973). The ability to demonstrate involvement of asymptomatic pathways had clear implications for the ability to grade the risk of development of disseminated MS, and was widely used in early diagnostic criteria. According to a consensus report produced in 2000 by the quality standards subcommittee of the American Academy of Neurology, visual evoked potentials were felt to be more valuable supporting evidence for the development of definite MS than sensory EPs, whilst there was insufficient evidence for the use of BSAEPs in this context

(Gronseth and Ashman, 2000). MRI, with its greater sensitivity to subclinical disease activity in all modalities except visual (Davies et al., 1998), has superseded EPs in a diagnostic capacity and this is reflected in modern diagnostic algorithms. The only EP to retain utility in the 2001 McDonald diagnostic criteria was the VEP and then only in the context of progressive disease from onset (McDonald et al., 2001). When these criteria were updated in 2005, EPs disappeared altogether from the algorithms (Polman et al., 2011; Polman et al., 2005).

Although their use in the diagnosis of MS is now redundant, evoked potentials have over the last 1-2 decades been gaining attention as a method to quantify the functional integrity of white matter tracts and by extension potentially perform as a surrogate for disability. Combining the different EPs to produce a multi-modal or global score has repeatedly shown correlation with clinical disability in both cross-sectional (Invernizzi et al., 2011; Leocani et al., 2006) and longitudinal analysis (Fuhr et al., 2001; Jung et al., 2008; Schlaeger et al., 2012b), and demonstrated an ability to predict the extent of progression of disability (Schlaeger et al., 2012a; Schlaeger et al., 2014b), all in mixed populations. Subsequent to initiation of the study reported in this thesis, a study looking at correlation of EPs with disability within a PPMS cohort has been published (Schlaeger et al., 2014a) though the cohort was small (just 22 patients with 6 failing to complete the study). Direct comparison with imaging surrogates was not undertaken.

Studies combining evoked potentials from different modalities have done so using a variety of techniques with none gaining pre-eminence. The measuring of amplitude of EPs is compromised by central amplification within the CNS, restricting assessment of axonal dysfunction or loss. Quantitative aspects of the latency of the EP are therefore integral and can be used alone (either transforming to an ordinal or continuous scale) or in conjunction with qualitative aspects of the recording. Ordinal scales have the advantage of smoothing the inherent fluctuation of neuronal function and convincingly accommodating absent potentials. The last point is a particular problem when using a raw EP or z-transformed (in which the raw score is converted to the number of standard deviations that it lies from the mean) latency. Methods to account for absent potentials include the use of quotients or average scores for summation of modalities (Schlaeger et al., 2014a), or direct replacement with the cohort maximum value (Fuhr et al., 2001). The correlations achieved by the different methods are largely comparable with a maximum reported Spearman correlation coefficient of 0.75 (Fuhr et al., 2001) achieved using continuous scale raw latency transformation of the MEP and VEP only.

Novel MRI parameters

In the context of active relapsing-remitting disease (although clearly related to inflammation) lesional metrics such as T1 persistent black holes (representative of tissue rarefaction following axonal injury) and magnetisation transfer ratio (a

measure of the transfer of nuclear spin polarisation which is elevated in white matter lesions (Filippi et al., 2013)) show promise for sensitivity to change in disability status both over time and in response to a treatment effect (Giacomini and Arnold, 2008). The reduction of inflammatory activity by powerful new therapeutics will inevitably compromise the ability of lesional metrics to reveal in-study neuroprotection and/or repair.

Given current concepts and understanding of the pathophysiology of MS, it seems intuitive that imaging modalities and sequences that seek to correlate with axonal dysfunction/loss are more likely to be able to capture the neurodegenerative element of the condition that underlies disability accrual, and consequently any neuroprotective effects (and beyond that tissue repair) of novel therapeutic agents. The most obvious of these is atrophy of CNS tissue; brain atrophy has been shown to correlate well with disability measures (Simon, 2006) and segmentation of brain volumes enables a degree of circumvention of some of the more obvious criticisms of this technique – namely susceptibility to confounds such as inflammatory tissue swelling, and increase in glial cell content. Studies have shown that perhaps counter-intuitively, grey matter atrophy is greater than that seen in white matter, at least early in the disease course (Tiberio et al., 2005). Atrophy of the spinal cord has shown even greater correlation with levels of disability ($r = 0.7$) in a mixed cohort of patients with MS (Losseff et al., 1996), thought to reflect the eloquence of affected pathways.

Advanced sequence imaging abnormalities of both white and grey matter that appears normal on standard sequences have provided important insights into the pathology of MS (Bakshi et al., 2008), and underscored a paradigm shift away from the traditional view of a 'disease of lesions' (Rice et al., 2013). Modalities used to assess normal appearing tissue include magnetisation transfer ratio (MTR), diffusion tensor imaging (DTI) and magnetic resonance spectroscopy (MRS).

- MTR reflects the magnetisation exchange between protons in tissues (either free or bound to macromolecules) and is decreased in tissue injury. Post-mortem studies have demonstrated clear correlation with both the degree of demyelination and percentage of axonal loss (van Waesberghe et al., 1999) and this translates to correlation with neurological physical disability (Hayton et al., 2009). Within a pure PPMS cohort normal appearing grey matter MTR has been shown to correlate with the requirement for a walking aid (Rovaris et al., 2008).
- DTI similarly correlates with axonal loss and demyelination (Mottershead et al., 2003). The technique can be used to generate measures of the direction (fractional anisotropy) and magnitude (mean diffusivity) of the Brownian motion of water molecules within tissues. Identification of white matter tracts can be achieved using the sequences, further enhancing the functional relevance of the parameters. Mean diffusivity (MD) shows an increase and fractional anisotropy (FA) a decrease in both normal appearing white and grey matter in patients with MS (Rovaris et al., 2005) and corticospinal tract measures of both correlate with disability measures (Tovar-Moll et al., 2015).

- Proton MR spectroscopy is able to measure *N*-acetyl-aspartate (NAA) levels, a correlate of neuroaxonal integrity. Studies in MS have revealed a reduction in levels in lesions and in normal appearing white matter that correlates with levels of disability (Sajja et al., 2009) and has underlined the hypothesis that progressive loss of axons is a key factor in the irreversible accumulation of disability in MS (Rocca et al., 2012). In PPMS total NAA levels in cortical grey matter have been shown to better correlate with EDSS than white matter levels (Sastre-Garriga et al., 2005).

Hypothesis

The hypothesis underlying this work is that bone marrow-resident stem cell populations have the capacity to limit injury and enhance repair within the CNS in human demyelinating disease, and that both post-mortem brain tissue and ante-mortem blood samples will yield evidence of mobilisation of these cells within the normal pathophysiology of the disease.

With respect to the quantification of disease status I hypothesise that neurophysiological parameters will provide better correlation with clinical measures of disease status and further support their use in the design of contemporary trials of neuroprotective and reparative agents.

Investigative strategy

The attempt at identification of cells of bone marrow origin was to be carried out in post-mortem brain samples obtained from patients with MS and in blood samples collected from patients attending the regional MS centre for clinical review;

- Identification of haematopoietic and mesenchymal cells within MS brain tissue using immuno-histochemical and -fluorescent techniques
- Identification of the same cell populations in blood samples using flow cytometric techniques

Interrogation of neurophysiological parameters as surrogates of disease status in PPMS would be carried out on data collected in an ongoing clinical project set up prior to the start of this thesis. A comparison with radiological parameters was planned and would entail recruitment of patients from the same cohort into an extension arm in which they would attend for an MRI scan and repeat clinical assessment.

Chapter 2

Bone marrow-derived stem cell detection in MS brain tissue

Introduction

Cells of bone marrow origin have for some time been hypothesised to contribute to spontaneous tissue repair in a range of diseases and a variety of tissues, including the central nervous system (Kørbling and Estrov, 2003). Initially, transdifferentiation to replace damaged cells was considered the principal mechanism of benefit. This was supported by studies of post-mortem brain tissue from sex-mismatched bone marrow transplants in which astrocytes and neurons of the donor gender constituted 1% of hippocampal neurons and astrocytes (Cogle et al., 2004).

In relation to disease of the brain however, there is now significant and widespread doubt concerning the ability of bone marrow cells directly to generate both neurones and myelinating oligodendrocytes. Whilst a certain amount of evidence exists for neural transdifferentiation of both mouse and human marrow-derived cells (Cogle et al., 2004; Eglitis and Mezey, 1997), paracrine reparative activities of bone marrow cells, including neuroprotection (Kemp et al., 2010), immune effects (Uccelli et al., 2007), and interactions with resident neural stem cells (Munoz et al., 2005) have been demonstrated and are thought to represent valuable potential

therapeutic effects in the context of neurodegenerative disease. Whatever the mechanism of benefit, certain bone marrow-derived cells clearly enter the peripheral circulation and then actively migrate into the damaged CNS (Kørbling and Estrov, 2003) - the ability of marrow-derived cells to infiltrate the CNS has repeatedly been demonstrated in both inflammatory and non-inflammatory conditions (Akiyama et al., 2002; Devine et al., 2003). In EAE, the murine model of Multiple Sclerosis (MS), human MSCs have demonstrated the property of disease abrogation in association with evidence of cellular infiltration of the CNS (Gordon et al., 2008), including specifically to white matter lesions (Gordon et al., 2008; Gordon et al., 2010). Mobilisation of bone marrow-derived cells, and specifically MSCs, is currently being explored as a potential therapy for MS. Little however, is known about their activity and fate *in-man* (as opposed to animal models). Further knowledge in this area would undoubtedly help in refining bone marrow transfer therapies for the disease.

No single marker exists that can reliably be used to identify MSCs though minimum criteria for their identification including patterns of surface markers (on a population basis) have been published, forming an accepted research standard (Dominici et al., 2006). CD271 or p75 neurotrophin receptor is a member of the tumour necrosis factor superfamily. It has been identified as a marker of primitive mesenchymal cells with greater clonogenic and differentiative capacity than MSCs isolated by plastic adherence (Quirici et al., 2002). Bone marrow-derived CD271-positive cells have been shown to possess neuroprotective properties within the

context of growth factor and nutrient withdrawal (Bakondi et al., 2009), and immunosuppressive properties (Kuçi et al., 2010). As well as expression by MSCs, CD271 or low-affinity nerve growth factor receptor is expressed by neuronal cells where it is thought to play a role in promoting survival and differentiation (Lv et al., 2014). It has not been identified in white matter, the location of classical demyelinating lesions in MS and therefore is a good candidate for identification of MSCs in brain tissue.

We aimed to establish whether bone marrow-derived cells incorporate into the multiple sclerosis lesion during the natural course of the disease and how they might integrate to facilitate endogenous repair.

Materials and Methods

Tissue for the study was provided by the UK Multiple Sclerosis Tissue Bank at Imperial College, London. Paraffin-embedded tissue from 14 neuropathologically confirmed cases of MS and 6 controls were available, pre-cut in 7µm sections.

Case	MS/Control	Age (years)	Sex (M/F)	MS phenotype	Disease duration (years)	Cause of death	Lesion type/Neuropathology
1	Control	93	F	n/a	n/a	Bronchopneumomia, cerebrovascular accident	Age-related changes only
2	Control	82	M	n/a	n/a	Unknown	Mild Alzheimer-type changes and features of possible ischaemia of the internal capsule
3	Control	71	M	n/a	n/a	Abdominal and retroperitoneal haemorrhage, ruptured spleen	Age-related changes and focal leptomeningeal infiltration of leukaemic cells
4	Control	88	M	n/a	n/a	Prostate cancer, bone metastases	Age-related changes, no metastatic disease
5	Control	84	F	n/a	n/a	Pancreatic cancer	Age-related and mild Alzheimer-type changes, no metastatic deposits
6	Control	82	M	n/a	n/a	Metastatic lung and liver cancer	Micro-metastasis in anterior caudate only

Case	MS/Control	Age (years)	Sex (M/F)	MS phenotype	Disease duration (years)	Cause of death	Lesion type/Neuropathology
7	MS	77	M	SPMS	39	Bronchopenumonia	Active
8	MS	56	F	SPMS	34	Septicaemia due to UTI	Active
9	MS	51	M	PPMS	2	Bronchopneumonia	Active
10	MS	35	F	SPMS	5	MS	Active
11	MS	45	F	SPMS	16	MS	Active
12	MS	64	M	SPMS	26	Bronchopneumonia, MS	Chronic active
13	MS	63	F	SPMS	26	UTI, MS	Chronic active
14	MS	55	M	SPMS	20	MS	Chronic active
15	MS	74	M	SPMS	37	Bronchopneumonia, MS	Chronic active
16	MS	44	F	SPMS	19	Urinary tract infection, MS	Chronic inactive
17	MS	53	M	SPMS	33	Septicaemia due to UTI, MS	Chronic active, chronic inactive
18	MS	83	F	SPMS	40	Septicaemia, bronchopneumonia, MS	Chronic inactive
19	MS	59	M	SPMS	39	Multiple sclerosis	Chronic inactive
20	MS	72	M	SPMS	43	Bronchopneumonia, MS	Chronic inactive

Lesion classification;

Lesions were first characterised using solochrome cyanine, myelin basic protein (MBP) and HLA DP/DQ/DR - 3,3'-diaminobenzidine (DAB) staining. For the purpose of solochrome cyanine staining, sections were first dewaxed and hydrated before staining in solochrome cyanine solution for 10 minutes, differentiation in 10% Iron Alum (aqueous) for 5 minutes and counterstaining with Kernechtrot solution for 15 minutes, all at room temperature with interspersed washes to intensify staining. Sections were then dehydrated and mounted. Sections were stained with antibodies to MBP (1:3200; Serotec, Oxford, UK) and HLA DP/DQ/DR (1/800; Dako, Denmark). Controls (control sections and MS sections without primary antibody incubation) were included in each run. Sections were first dewaxed and hydrated, and then immersed in 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Antigen retrieval was performed with repeated heating in sodium citrate buffer (0.01 M, pH 6.0, 5 min) or Ethylenediaminetetraacetic acid (EDTA) buffer (1 mM, pH 8, 10 min) as appropriate and rinsed in phosphate-buffered saline (PBS). Non-specific binding was blocked by 20-minute immersion in Vectastain blocking serum (Vector Laboratories, Peterborough, UK). Primary antibodies were diluted in 3% BSA/0.1% Azide and sections incubated for 18 hours at 4^{o}C. The sections were then rinsed in PBS before incubation for 20 min with secondary antibody (Vectastain Biotinylated Universal antibody) and 20 min with Vectalite ABC complex (Vector Laboratories, Peterborough, UK). DAB and 0.01% H2O2 was then added to the sections for 10 minutes before washing and immersing in copper sulphate DAB enhancer (4 min).

Cell nuclei were counterstained with hematoxylin and the sections dehydrated, cleared and mounted.

Immuno-fluorescent labelling of paraffin-embedded sections;

Sections were dewaxed, hydrated and washed as above. Sections were incubated for 1 hour in 5 mM copper sulphate and 50 mM ammonium acetate at room temperature to block auto fluorescence. Antigen retrieval was then performed by repeated heating in sodium citrate buffer (0.01 M, pH 6.0, 5 min). Sections were incubated for 1 hour at room temperature in 10% normal goat serum (NGS) diluted in PBS containing 0.1% triton to block non-specific antibody binding and then in the primary antibodies diluted in 10% NGS/PBS for 18 hours at 4^{o}C. Cell characterisation using double immunofluorescence was performed using a number of cell markers in varying combinations. These consisted of antibodies to MBP and HLA DP/DQ/DR (1:400) as above, Iba-1 (1:250), NG2 (1:500), GFAP (1:5000), CD3 (1:250), CD68 (1:800), CD34 (1:250), CD45 (1:100), CD73 (1:250), CD90 (1:), CD105 (1:200), CD271 (1:400) (all Abcam, Cambridge, UK) and Stro-1 (1:200; Millipore, Massachusetts, USA). Sections were then washed in PBS and incubated for 30 min in the dark with the appropriate species-specific secondary Alexa Fluor 488/555 antibodies (1:500; Invitrogen, Paisley, UK). After a further wash sections were washed in PBS and mounted in Vectashield medium containing the nuclear dye 4'6'-diamidino-2-phenylindole (DAPI). Imaging was performed using an inverted Leica CTR 6000 fluorescence microscope and merged with Leica Application Suite Advanced Fluorescence software.

Stro-1/CD271 immunoperoxidase labelling of paraffin-embedded sections;

Sections were prepared as for MBP and HLA DP/DQ/DR labelling for the purposes of lesion characterisation. Sections were labelled with anti stro-1 (1:800; Millipore, Massachusetts, USA) or anti-CD271 antibodies (1:400; Abcam, Cambridge, UK), incubated for 18 hours at 4^{o}C. Antibody was labelled with DAB using the Vectastain Universal Kit. Acquisition of images was performed using an Olympus IX70 microscope (Olympus, UK) and Image Pro-Plus software (Bethesda, USA). Cell quantification was performed with the aid of Image-J software (NIH, USA).

Statistical analysis;

Statistical analysis of the data was carried out using a regression model that enabled adjustment/correlation for multiple sections taken from the same patient/brain (cluster option). Non parametric bootstrap analysis was used to estimate standard errors and 95% confidence intervals to account for potential non-normality of data. The statistical analysis was carried out using STATA v12 (StataCorp, TX, USA) and graphs generated using GraphPad PRISM 5™ (Graph Pad Software, CA, USA). Values of $p<0.05$ were considered statistically significant.

Results

Lesion classification;

Lesion identification and activity was first established in the paraffin-embedded sections. Areas of demyelination were readily identifiable using solochrome cyanine staining and immunolabelling for MBP within the sections derived from patients with MS. No evidence of demyelination was seen in any of the control samples. Sections derived from the patients with MS were immunolabelled with antibody to the macrophage/microglial markers HLA-DP/DQ/DR allowing classification of demyelinated lesions as active, chronic active or chronic inactive, according to levels and distribution of HLA-DP/DQ/DR positive cells. In active lesions microglia are seen throughout the area of demyelination; in chronic active lesions there is a rim of activated microglia at the periphery of areas of demyelination with cells demonstrating typical thickened ramifications - infrequent positive cells are also seen within the body of the lesion; in chronic inactive lesions there is sparse distribution of microglia throughout the lesion. In non-demyelinated white matter adjacent to areas of demyelination HLA-DP/DQ/DR positive cells were also seen in an evenly distributed but less numerous pattern. Minimal or no positive cells were identified in the white matter in the sections from control subjects. 6 cases containing active lesions, 5 containing chronic active and 5 cases containing chronic inactive lesions were stained for cell enumeration.

Figure 2.1; Representative histological sections from a control subject showing intact white matter and minimal evidence of infiltration of activated microglia; **A, D** - solochrome cyanine stain (blue) identifies white matter; **B, E** - DAB-immunolabelled (brown) Myelin Basic Protein identifies myelinated white and grey matter; **C, F** - DAB-immunolabelled (brown) HLA-DP/DQ/DR identifies activated microglia. Magnified images (**D**-**F**) are taken from within the same region of white matter identified by red broken box in **A-C**. Nuclei stain pink (kernechtrot) in **A** and **D** and blue (haematoxylin) in **B, C, E** and **F**. Scale bar = 50µm.

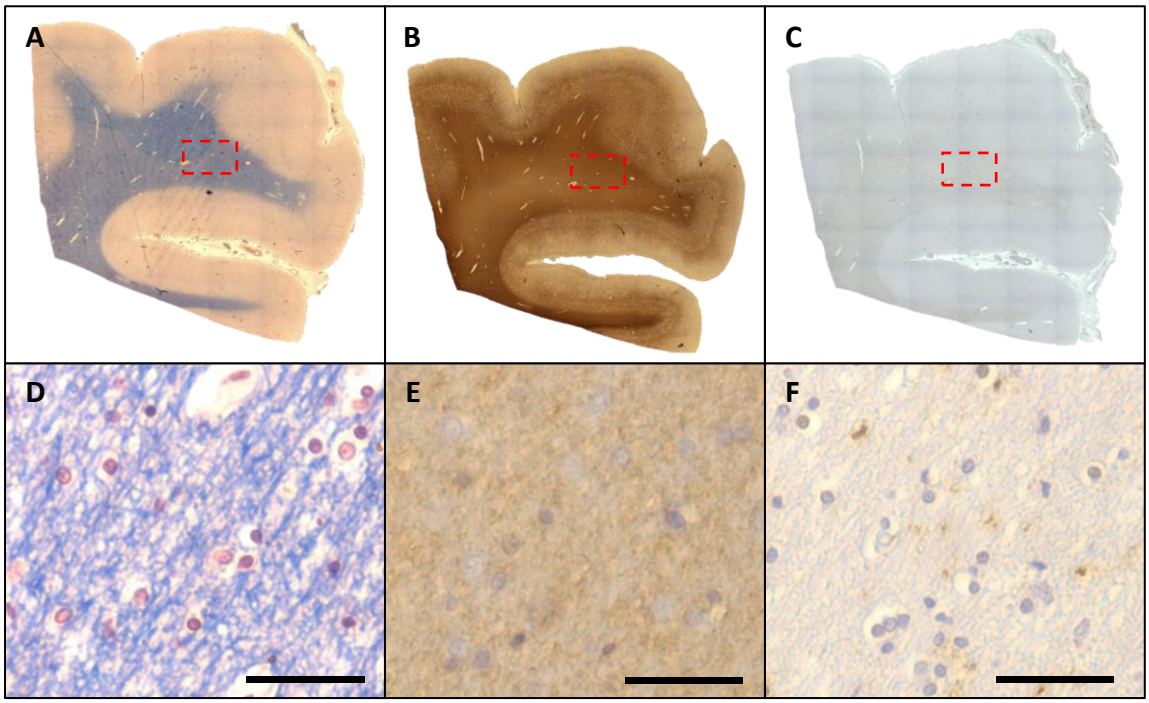
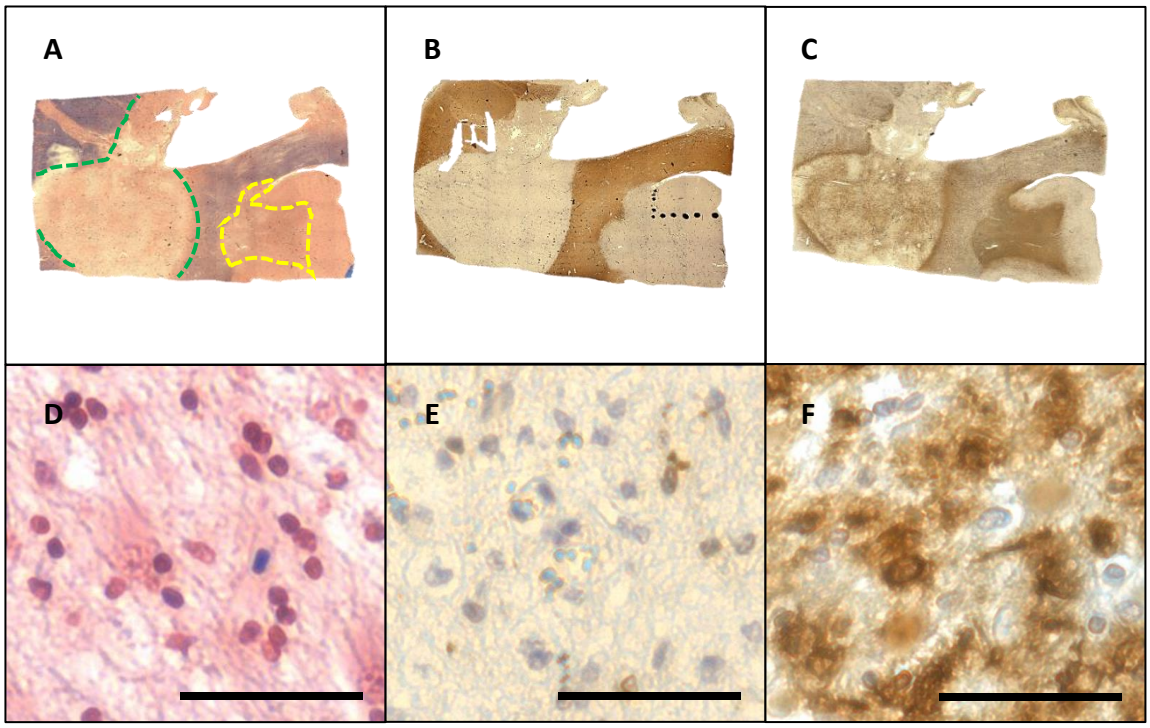


Figure 2.2; Representative section from patient with MS demonstrating an active and chronic active lesion. Sections labelled with solochrome cyanine, MBP and HLA-DP/DQ/DR are displayed as in **Fig 2.1**. Chronic active lesion (outlined with green broken line in **A**) is denuded of myelin and has a rim of activated microglia at the periphery of the lesion (identified with HLA-DP/DQ/DR immunolabelling in **C**). Active lesion (outlined with yellow broken line in **A**) is similarlay denuded of myelin but shows diffuse infiltration of activated microglia as seen in **C**. Magnified images are of the active lesion. Note presence of activated microglia in **F**. Scale bar = 50µm.



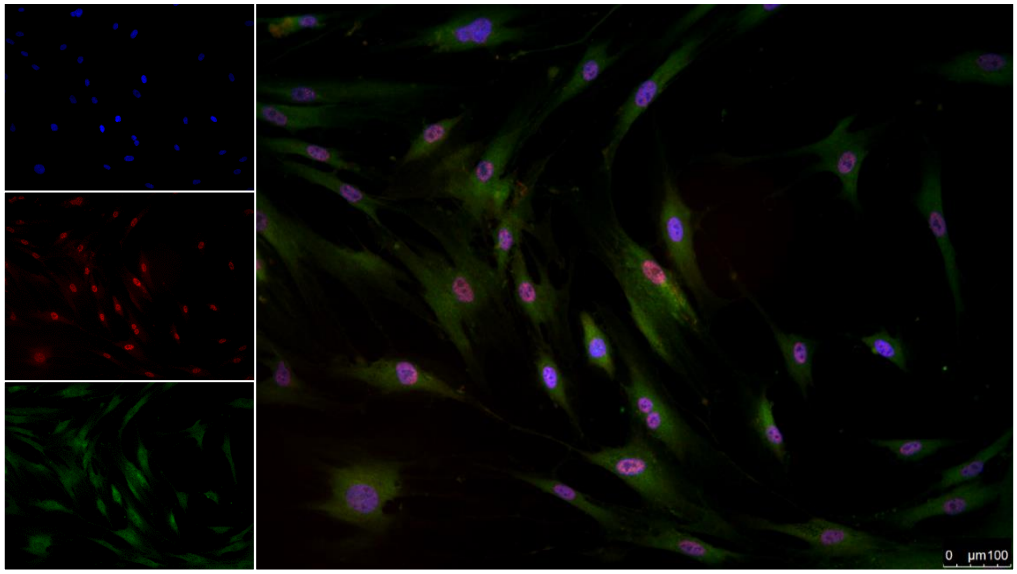
Identification and characterisation of CD271-positive cells;

Millipore anti-CD271 antibodies

Prior to staining of MS brain sections, MSCs cultured by plastic adherence were immunolabelled as a positive control. Initial work was performed using Rabbit anti-CD271 IgG produced by Millipore. Cultured MSCs were readily co-labelled with anti-CD271 and anti-CD105 antibodies (*see fig 2.3a*). The CD271 immunolabels had a perinuclear predominance with less marked staining in the cell body. Anti-CD105 produced homogenous cytoplasmic staining and the control samples (with no primary antibodies) showed absence of staining (*fig 2.3c*).

Figure 2.3; Cultured MSCs demonstrated co-expression of CD271 and CD105; CD271 is expressed in a perinuclear distribution. CD271 stained red (Alexa-Fluor 555) and CD105 green (Alexa-Fluor 488); nuclei are blue (DAPI). **B** represents magnified 555 channel from **A; C** is negative control (fluorescent secondary antibodies only).

A



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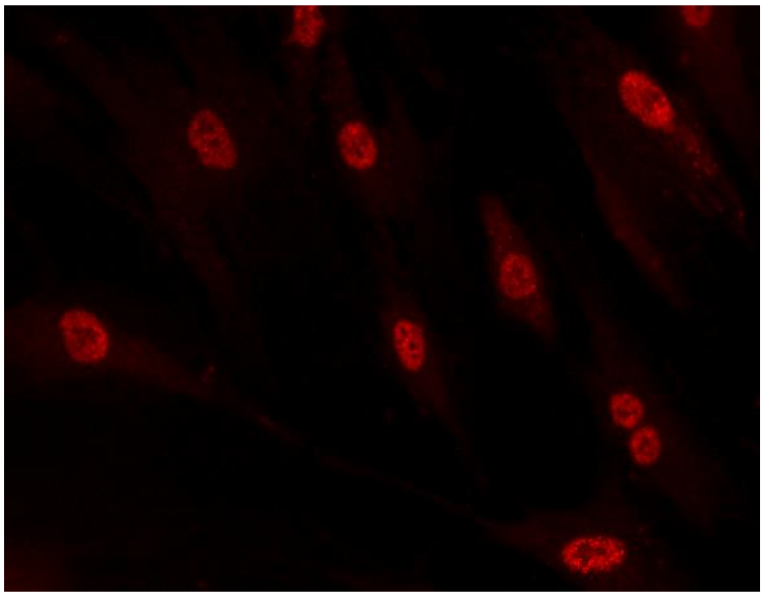
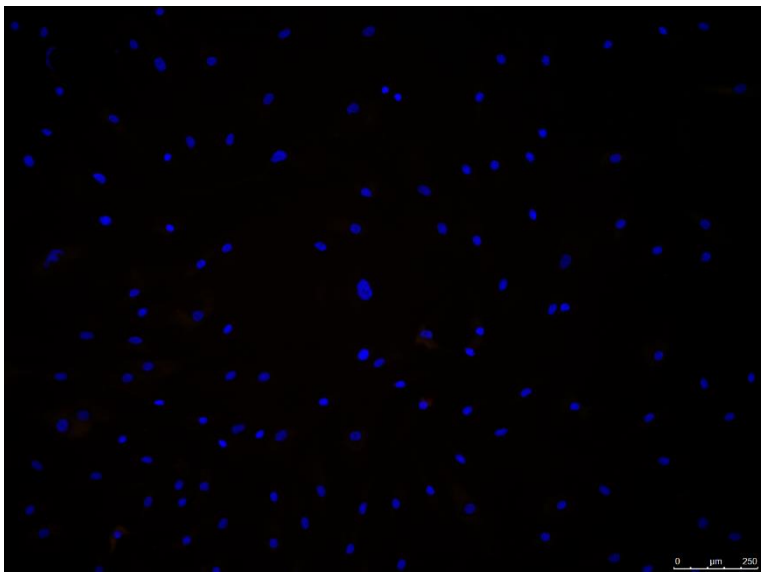


C



To determine whether CD271-positive cells were present in brain sections from patients affected by MS we screened DAB immunolabelled examples of each of the lesion-types (active, chronic-active and chronic-inactive). Linear structures redolent of elongated processes were visible in areas with little MBP staining that may have represented denuded axons (*fig 2.4c*). In the active lesions and at the periphery of chronic-active lesions, cells with small cytoplasms which did not relate to areas of MBP staining were visible (*fig 2.4d*).

Further characterisation of these cells was performed using double immunofluorescence. The cells were negative for a number of markers found on cell types known to be prevalent in demyelinated MS lesions including; CD3 (T-lymphocytes), CD45 (leucocyte common antigen), CD68 (Macrophages), Iba-1

(activated Microglia), GFAP (astocytes), MBP (mature oligodendrocytes) and NG2 (oligodendrocyte precursor cells).

Figure 2.4; Cells staining positive for CD271 were prevalent in areas of active inflammation. **A** HLA-DP/DQ/DR DAB-immunolabelled (brown) section from patient with MS and **B** corresponding section labelled with anti-CD271 antibody. **C** and **D** are taken from region of interest outlined by broken red line in **B**. Scale bar = 50µm.

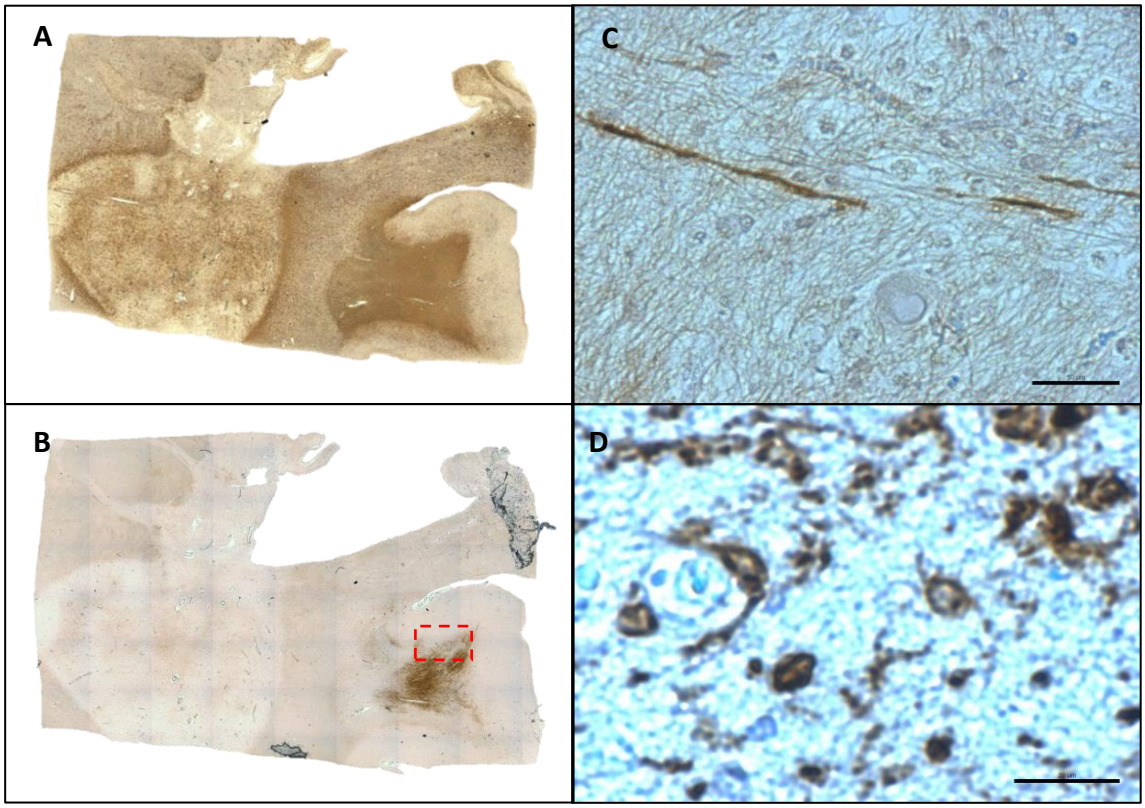
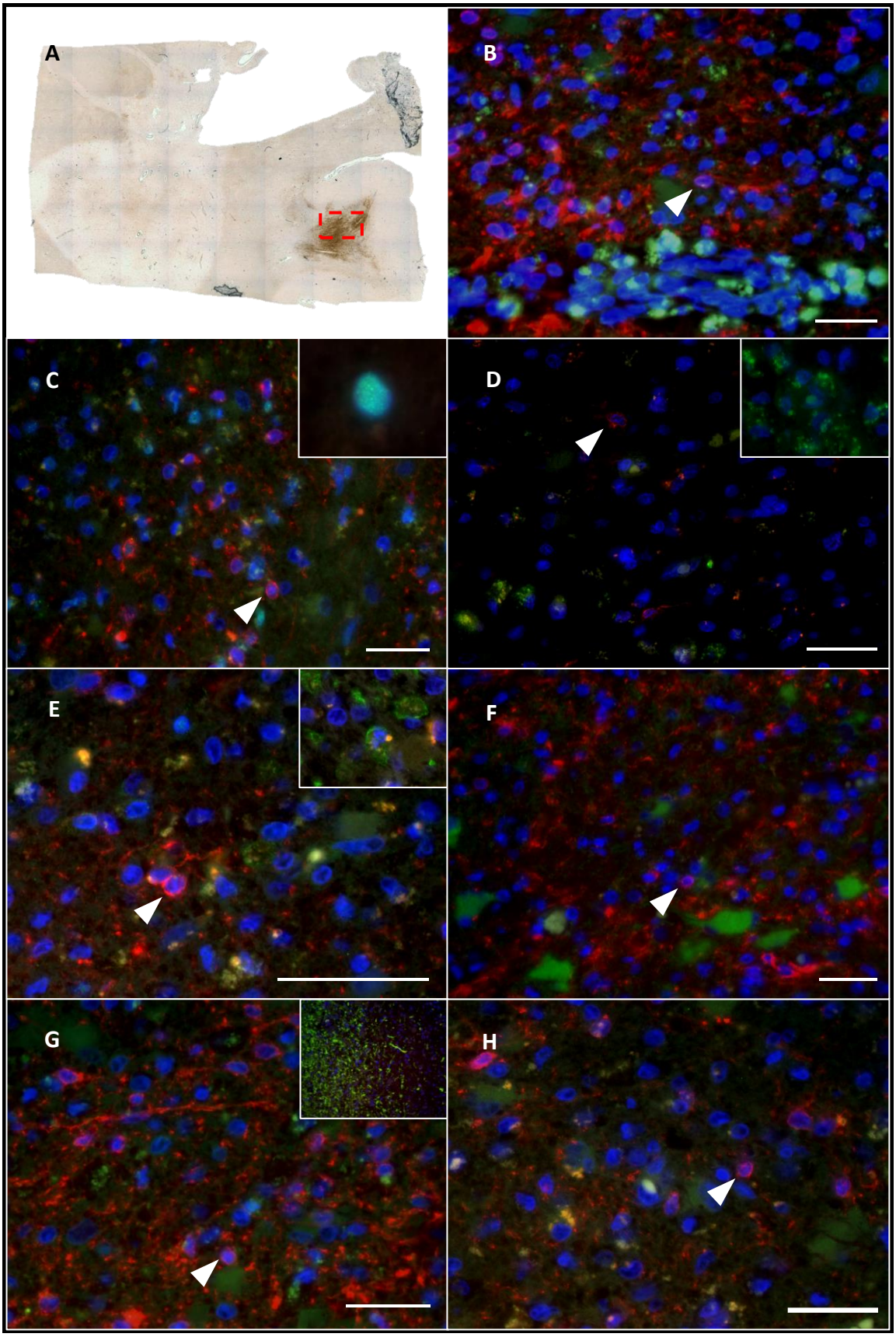


Figure 2.5; Cells staining positive for CD271 did not display colabelling with any of a plethora of cell markers. Immunofluorescent images (**B**-**H**) taken from area of 271 DAB-immunolabelled staining (brown) outlined by red broken line in **A**. CD271 is labelled with Alexa-Fluor 555 (red) and the co-label marker (**B**-CD3; **C**-CD45; **D**-CD68; **E**-Iba-1; **F**-GFAP; **G**-MBP; **H**-NG2) with Alexa-Fluor 488 (green) in all images. White arrow heads identify cells that stain positive for CD271 but not for the co-label. Where not apparent in the main image, small inserted images demonstrate positive internal control staining.

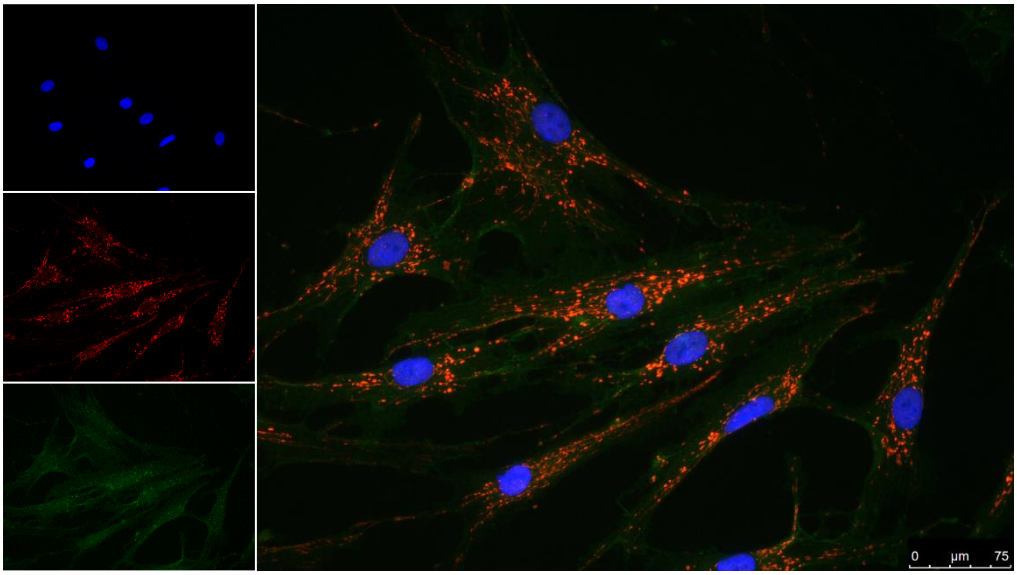

A
B
C
D
E
F
G
H

Abcam anti-CD271 antibodies

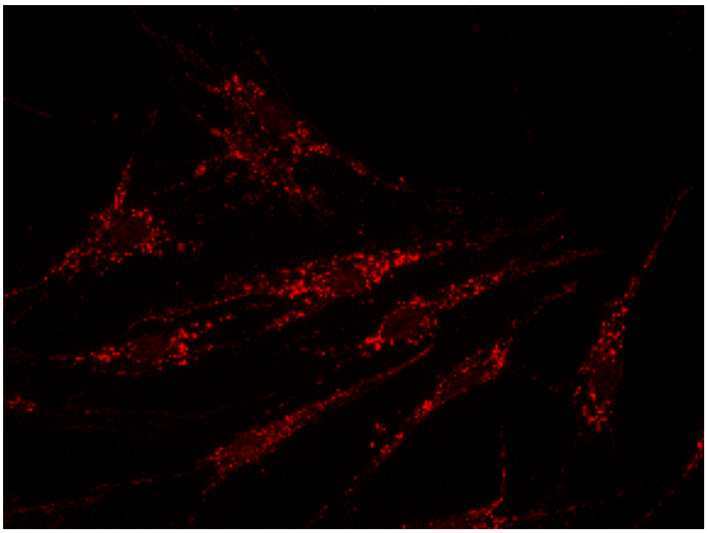
I next labelled laboratory-cultured MSCs with an alternative anti-CD271 antibody due to failure of inhibition of staining with Millipore antibodies with an Abcam CD271 peptide block. The MSCs again readily co-labelled with anti-CD271 and anti-CD105 antibody (*Fig 2.4a*). In contrast to the Millipore antibodies, staining with anti-CD271 was in a heterogenous cytoplasmic pattern with a slightly lobulated matrix-like appearance and without the previously witnessed perinuclear preponderance. CD105 labelling again produced the homogenous cytoplasmic pattern of staining and the negative control (incubated with PBS instead of primary antibodies) didn't display any fluorescence (image not shown).

Figure 2.6; Cultured MSCs demonstrated co-expression of CD271 and CD105; CD271 distribution was different to that seen using Millipore antibodies (see **Fig 2.3**). CD271 is stained red (Alexa-Fluor 555) and CD105 green (Alexa-Fluor 488); nuclei are blue (DAPI). **B** represents magnified 555 channel from **A**.

A



B



Anti-CD271 DAB-immunolabelling of the different types of MS lesions was then performed, revealing positive cells in areas of inflammatory activity (*Fig 2.7*) – namely active lesions and the periphery of chronic active lesions. Labelling of the cells was confirmed using immunofluorescence. Furthermore the labelling was inhibited by the use of a CD271 peptide block (*Fig 2.8*). Further cell characterisation was performed using double immunofluorescence which demonstrated co-expression of CD271 and Stro-1 (*Fig 2.9*). Stro-1 cellular expression was confirmed using DAB-immunolabelling which demonstrated positive cells in correlation with CD271-expressing cells in areas of inflammatory activity.

Fig 2.7; Cells expressing CD271 were readily identifiable in areas of active inflammation. Image shows DAB-immunolabelled (brown) CD271 positive cells; nuclei counterstained with haematoxylin (blue). Scale bar = 50µm.

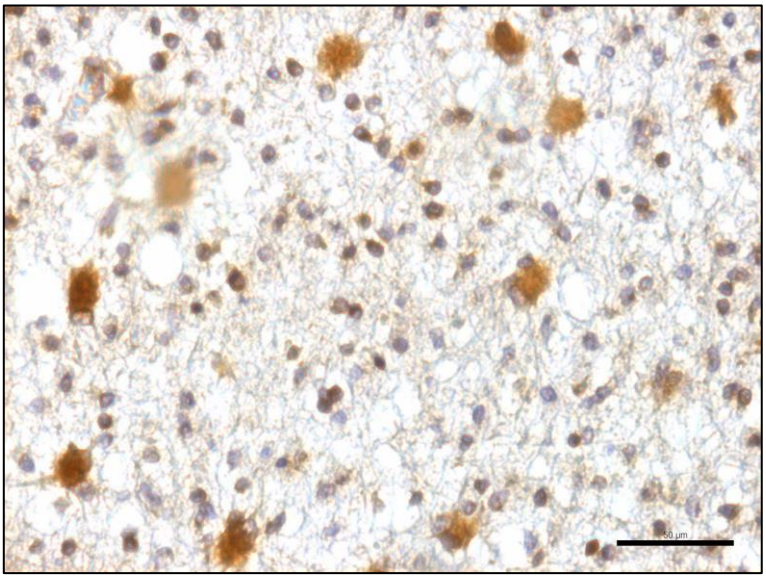


Fig 2.8; A CD271-positive cells are also identified using immunofluorescence and **B** staining is inhibited using CD271 peptide block. CD271 labelled with Alexa-Fluor 555 secondary antibodies (red). Image insert in **A** represents magnified example of CD271-positive cell.

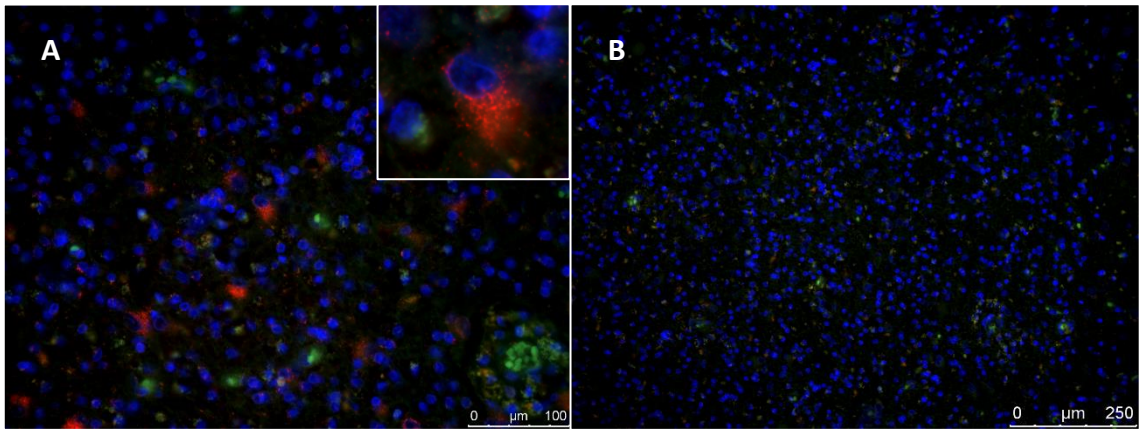
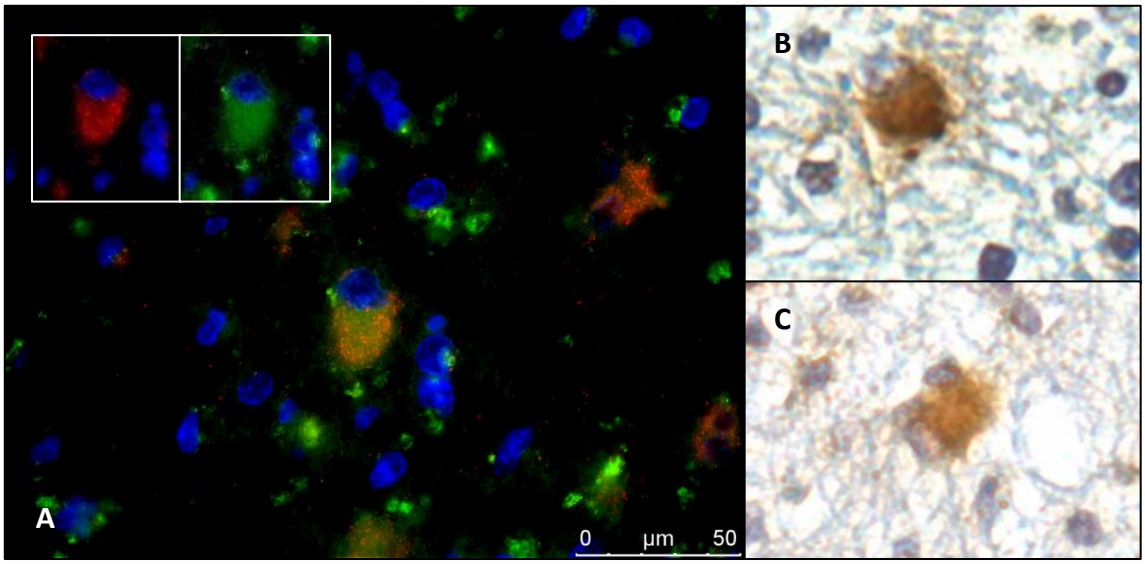


Figure 2.9; CD271-positive cells found in areas of active inflammation within MS lesions are also Stro-1-positive. **A** Double immunofluorescence demonstrating co-labelling of CD271 (red) and Stro-1 (green). DAB-immunolabelling confirmed the presence of **B** Stro-1-positive and **C** CD271-positive cells.

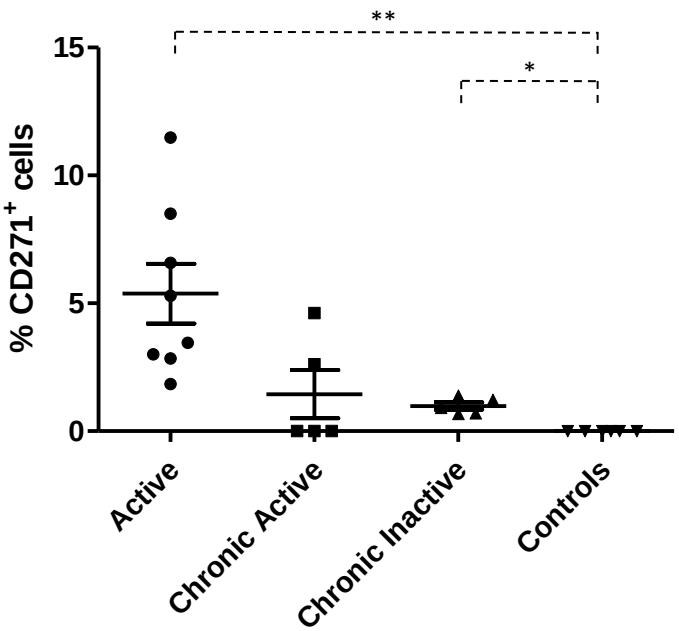


Cell quantification;

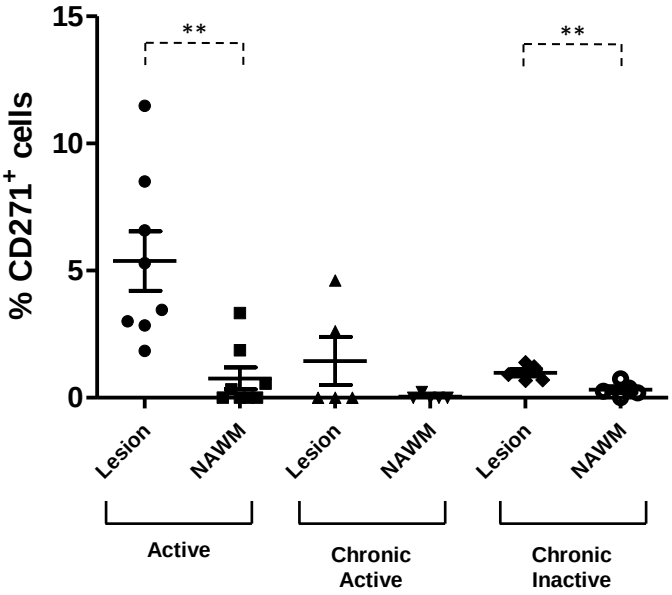
$CD271^+$ cell numbers and proportion within the different types of lesions were next quantified. Regions of interest were identified manually using MBP- and HLA-DP/DQ/DR labelled adjacent sections. Cell numbers were counted in a 320µm x 240µm area (0.0768mm^2) area selected at random under low resolution. Data was acquired in triplicate at x20 magnification. Cluster option modelling was used to adjust for multiple sections from the same subject. Overall the number of cells were higher in the areas of active inflammation, whether this was lesion-wide (as in active lesions) or in an active periphery (as found in chronic active lesions). Within the infiltrating cell cohort in the active lesion a significantly higher percentage of CD271-expressing cells was found compared with control samples, chronic inactive and chronic active lesions. We also found a higher proportion of $CD271^+$ cells when comparing with normal-appearing white matter from the same section in both the active and chronic inactive lesions but not in the chronic active lesions. Finally within the chronic active lesions the presence of $CD271^+$ cells mirrored that of activated microglia with a higher proportion found at the active rim compared with the lesion as a whole or indeed adjacent NAWM. The proportion seen in areas of active inflammation at the periphery of the lesions was not statistically different from that in active lesions.

Fig 2.10; A Proportion of CD271^{+} cells is higher in active lesions when compared with control sections. There is also a higher proportion in the chronic inactive lesions; **B** Proportion of CD271^{+} cells in active, chronic active and chronic inactive sections compared with normal-appearing white matter (NAWM) from the same tissue samples; **C** Proportion of CD271^{+} cells is higher in the active rim of the chronic active lesion compared with NAWM though not the lesion as a whole. There is no statistically significant difference from the proportion identified in the active lesions.

A



B


15
10
5
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Lesion
NAWM
Lesion
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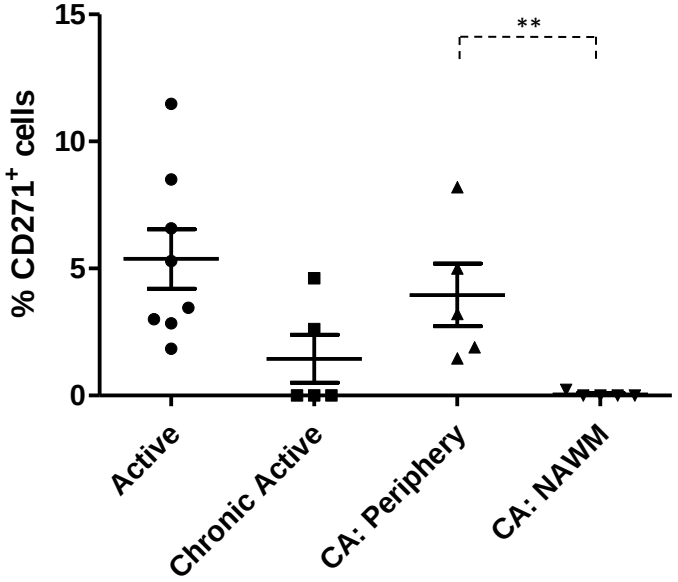
Active
Chronic Active
Chronic Inactive

C


15
10
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% CD271+ cells
**
Active
Chronic Active
CA: Periphery
CA: NAWM

Discussion

Here, we have demonstrated the presence of cells expressing CD271 and Stro-1, two markers that are expressed by multipotent mesenchymal stromal cells, within areas of active inflammation in brain samples taken from patients with MS. The cells did not express markers seen on a multitude of inflammatory and resident glial cells that constitute most previously identified cells within white matter MS lesions. Cell numbers are clearly highest in active lesions but were also seen in chronic active and even chronic inactive lesions to a greater extent than control brain tissue. Furthermore, adjacent to areas of immune activity, in so-called normal-appearing white matter (in which myelin is intact), the presence of diffuse activated microglia was accompanied by the presence of more of these cells than were seen in control subject white matter.

The frequency of lesion-based Stro-1/CD271^{+} cells identified in this work was higher than might have been intuitively predicted but corroborative evidence in the literature is lacking - as far as we are aware no group has previously sought to identify cells expressing these markers in human post-mortem tissue despite several studies identifying MSC migration into the CNS in EAE (Gordon et al., 2008; Gordon et al., 2010). No plausible alternative explanation for the presence of these Stro-1/CD271^{+} cells was identified in this work - co-localisation of various markers of resident glial and immune cells was not demonstrated. The changes in cell

surface markers and or differentiation of MSCs following introduction in humans is unknown. Whilst the cells did not display markers such as CD90 and CD105, it remains a possibility that expression has been downregulated *in-vivo*.

The precise function of Stro-1 is unknown. It is a 75kd single pass type I protein that has been shown to translocate from the endoplasmic reticulum to the cell membrane in response to intracellular calcium depletion (Barkhordarian et al., 2011). Its use as a marker for MSCs is well established. Whilst Stro-1 is also expressed on endothelium and has been identified in various organs including but not limited to the liver, lung and kidney (Lin et al., 2011; Ning et al., 2011), the cells identified in this work were not found in close proximity to vascular structures.

The potential identification of MSCs in the CNS of human subjects with MS would be consistent with a wide body of evidence from animal studies. It is well established that bone marrow-derived stem cells are able to migrate and infiltrate sites of tissue injury in animal models of neural insult (Chopp and Li, 2002; Mahmood et al., 2003; Mahmood et al., 2005) including in inflammatory pathology such as EAE (Gordon et al., 2010), the murine model of MS. Despite the problem of lung sequestration of intravenously delivered cellular therapies (Fischer et al., 2009) MSCs have been identified in the CNS after such delivery (Gerdoni et al., 2007; Kassis et al., 2008; Zhang et al., 2006) with several authors postulating that local infiltration is a prerequisite for neuroprotection (Kassis et al., 2008; Zhang et

al., 2006). Indeed MSCs were even found to be present in the CNS of mice with EAE when administered via the intra-peritoneal route (Gordon et al., 2008).

The neurobiology of this migratory capacity has been extensively studied with the identification of multiple chemotactic molecules able to promote MSC infiltration of damaged tissue (Honczarenko et al., 2006; Kitaori et al., 2009; Lee et al., 2006; Ringe et al., 2007). These include the growth factors platelet-derived growth factor AB (PDGF-AB) and insulin-like growth factor 1 (IGF-1) (Ponte et al., 2007) and chemokines including stromal-derived factor 1 (SDF-1) (Ponte et al., 2007; Yu et al., 2016). Furthermore preincubation of MSCs with specific inflammatory cytokines has been shown to upregulate CXCR4 expression and enhance SDF-1-mediated cell migration suggesting a preconditioning function of the systemic inflammatory response (Yu et al., 2016).

The relevance of these findings to the use of MSCs as a potential therapy for protection and repair in MS is clear. Whilst our understanding of MS as a diffuse brain disease rather than a 'disease of lesions' has led to a refocus on the concept of remote or systemic effects of these cells, direct infiltration of lesions and brain parenchyma more widely would provide a more intuitive explanation for their hypothesised benefit. Indeed many have already suggested that this is a prerequisite for effective protection and repair of neurons. Evidence of this occurring in human subjects with MS has not previously been reported. Given the

inherent problems associated with cell marker plasticity, until biological labels that are safe in human use are developed the question of MSC infiltration of the brain in humans is likely to remain to be definitively answered.

Chapter 3

Bone marrow-derived stem cell detection in Multiple Sclerosis

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Introduction

Bone marrow has traditionally been thought of as containing haematopoietic stem cells (HSCs) and stromal 'support' cells including multipotent mesenchymal stromal cells (MSCs). Bone marrow-derived cells have been shown to have reparative potential in several neurological diseases including multiple sclerosis and clinical trials are in progress (Rice et al., 2013).

CD34 is a cell surface marker widely used in clinical bone marrow transplantation to identify HSCs capable of repopulating the bone marrow after myeloablative therapy. Release of $CD34^+$ haematopoietic stem cells (HSC) and the $CD133^+$ subset (associated with high proliferative (de Wynter et al., 1998) and differentiation potential (Torrente et al., 2004)) into the peripheral circulation has recently been demonstrated in patients receiving anti-CD49d monoclonal antibody (Natalizumab) for the treatment of multiple sclerosis (Bonig et al., 2008; Jing et al., 2010; Zohren et al., 2008). Potential mechanisms of repair and/or remodelling of non-haematopoietic tissue by haematopoietic stem/progenitor cells have been demonstrated (Gordon et al., 2006). Whether these cells contribute to disease

modification and tissue repair in MS is not currently known but suggests that the monoclonal antibody may have treatment effects beyond immunosuppression.

No single cell surface marker has been identified that reliably isolates MSCs. These cells are characterised by the presence and absence of expression of multiple cell surface markers, adherence to plastic in vitro and potential to differentiate into fat, cartilage and bone (Dominici et al., 2006). Isolation and identification of these rare cells in the peripheral circulation has been challenging, usually being achieved in the context of administration of granulocyte colony-stimulating factor (G-CSF) (Kassis et al., 2006) or isolation of small cell subsets obtained through centrifugation of large volumes (up to 500ml) of peripheral blood (Zvaifler et al., 2000).

Flow cytometry is a powerful tool enabling analysis of cell physical and chemical properties on an individual cell basis. Early impedance-based devices used the Coulter principle to measure cell/particle size whilst modern fluorescence-based devices use specific electromagnetic wave-length excitation and emission to identify antibody/molecule-conjugated fluorochromes. Advances in affinity reagent technology, particularly monoclonal antibody development, in parallel with technological advances and data analysis techniques have enabled the development of multiparameter or polychromatic flow cytometry in which multiple cell markers and physical characteristics can be analysed simultaneously. The identification of cell populations through expert manual partitioning or 'gating' of

cell populations is performed visually using 1 or 2-dimensional plots of repeated 'events' ie the measured parameter of each cell that has been analysed. The use of multidimensional plots enables simultaneous visual analysis of several different cell parameters (in this instance, cell surface markers) with clear implications in rare event detection. This technology has not been applied to detection of MSCs in the peripheral blood stream.

Detection of the presence of bone marrow-derived stem cells in the peripheral circulation would provide evidence for their mobilization, supporting the theory of migration from bone to brain, and identifying potential therapeutic mechanisms of clinically available drugs. $CD34^+$ cell mobilization in patients receiving anti-CD49d monoclonal antibodies for the treatment of MS has already been established (Bonig et al., 2008). Higher levels of circulating $CD34^+$ cells were accompanied by a lower proportion of $CD133^+$ $CD34^+$ cells, though an elevation in their overall numbers. It is possible these cells are contributing to the treatment effect of the drug which also has powerful anti-inflammatory functions, impairing trafficking of mature lymphocytes. Circulating levels of these cells in patients treated with other pharmacological agents, including alemtuzumab have not yet been quantified, nor has work looked at the different subsets of disease phenotypes, or at alternative circulating bone marrow-derived stem cell populations.

The ability to identify stem cells at small numbers in the peripheral circulation will also have significant benefits for the monitoring of stem cell populations during

systemically administered therapies for a wide range of neurological and non-neurological conditions.

In this work we aimed to determine whether HSCs are mobilised during an acute MS relapse or by treatment with disease-modifying drugs (DMDs) other than Natalizumab. We also aimed to develop a novel multichannel flow cytometric technique to enable identification of MSCs and to quantify their release under the same conditions.

Materials and Methods

Identification of Haematopoietic Stem Cells;

Blood samples were collected from consenting patients and control subjects attending a regional MS centre for the purposes of ongoing clinical care. Relapses and MS phenotype were identified by the treating neurologist with identification of disease-modifying therapies from participant's records. Samples were collected from MS patients (n=67) and control subjects (n=6) who did not have MS or other neurological illness. Of the 67 patients with MS, 56 had relapsing-remitting MS, 4 had primary progressive MS and 7 had secondary progressive MS. The breakdown of those with relapsing-remitting disease was as follows;

	Remission	Relapse
No DMD	7	5
Beta-interferon	9	2
Copaxone	5	0
Fingolimod	1	0
Natalizumab	10	3
Alemtuzumab	6	0

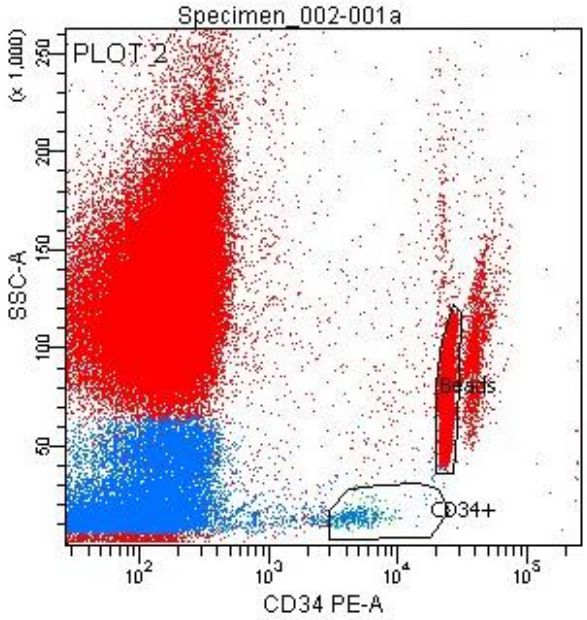
Relapse samples were taken prior to the administration of corticosteroids and samples from those receiving Natalizumab or Alemtuzumab prior to infusion. Procurement of samples was in accordance with Research Ethics Committee approval following the acquisition of informed consent.

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) by MS nurse practitioners at the regional MS centre and stored at 4^{o}C. Samples were processed within 72 hours to avoid cell loss or alteration to patterns of cell surface marker expression. 50µl of blood was incubated for 15 minutes at 21^{o}C with 10µl anti-CD45 (BD Biosci 345808 FITC) and anti-CD34 (BD Biosci 345802 PE) antibodies as well as 5µl anti-CD133 (Miltenyi 130-090-826 APC). 450µl of red cell lysis buffer (BD FACS lysing solution 06411) was then added and left for 10 minutes until the suspension became transparent. The sample was added to a BD Trucount tube containing microbeads which allow determination of absolute cell number and concentration. The sample was then diluted with phosphate buffered saline and processed using a BD FACSCanto flow cytometer.

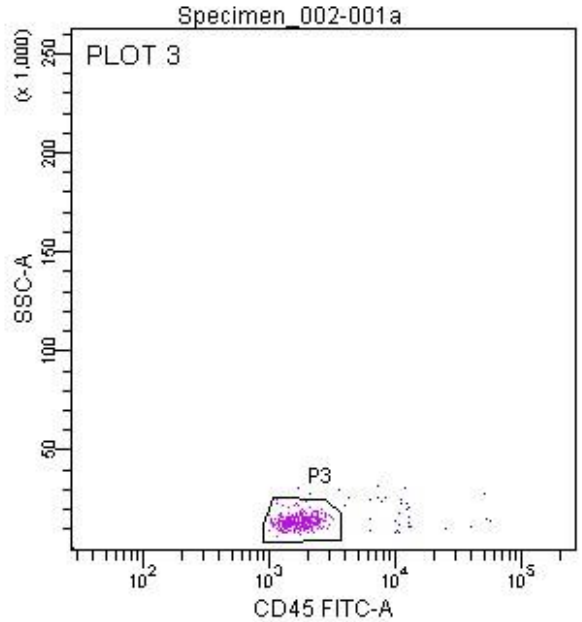
The following gating strategy is in routine clinical use and generated a $CD34^+$ cell count ($cells\mu l^{-1}$), $CD133^+$ cell count ($cells\mu l^{-1}$) and the percentage of $CD34^+$ cells that also expressed CD133.

Figure 3.1; Scatter plots demonstrating the gating strategy for the identification of $CD34^+$ and $CD133^+$ cells using side scatter (SSC-A), CD34, CD45 and CD133 expression. Each plotted dot represents a single cell.

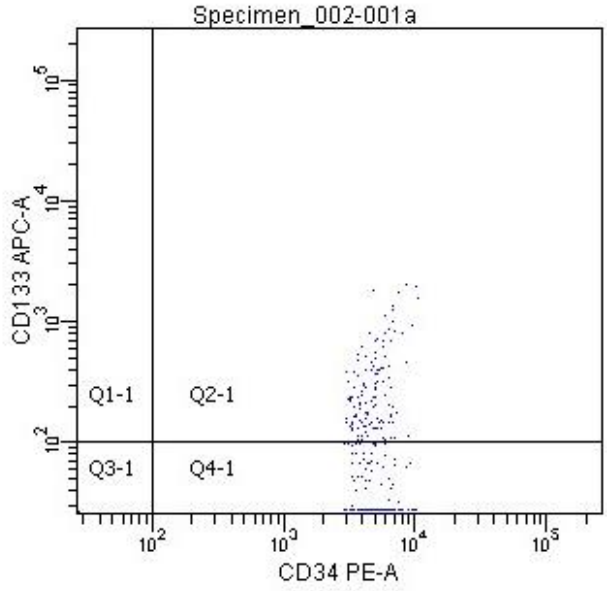
A Identification of CD34-positive low granularity cell population



B $CD34^+$ HSCs are $CD45^-$ - cells represented are the gated population in **A**



C Partitioning of CD34 cells into CD133-positive and negative subsets (note logarithmic scale y-axis) – the scatter plot demonstrates a heterogeneous cell population which are taken from the gated population in **B**



Data was analysed using GraphPad Prism software. The use of parametric tests was only applied where statistical normality of the data was confirmed. 1 way ANOVA and unpaired t-tests were used to identify differences in cell densities between groups with post-hoc testing using Dunnet's multiple comparison test. All t-tests give 2-tailed p values.

Identification of Mesenchymal Stromal Cells;

Bone marrow, with its higher numbers of resident MSCs was used for the development of the multichannel flow cytometry algorithm. Bone marrow was

obtained as per ethics committee approval whereby patients consented to the use of waste products from a primary total hip replacement. 2-5ml of liquid marrow was collected in 10ml RPMI/10% heparin by the orthopaedic surgeons performing the operation in the Avon orthopaedic centre. The marrow sample was stored at 21^{o}C and processed within 24 hours. The red cell population was removed by the addition of 20ml of Ammonium Chloride lysis buffer (0.15M ammonium chloride, 0.01M potassium bicarbonate and 0.15mM EDTA in ddH2O). The suspension was then centrifuged at 1800rpm for 10 minutes and washed in phosphate buffered saline. The cells were then suspended in PBS at $2x10^{6}$ cells ml^{-1} and incubated for 10 minutes at 21^{o}C with 5µl each of 7 fluorochrome-conjugated antibodies;

CD90-FITC

CD11b-PE

CD34-V450/brilliant violet

CD271-APC

CD105-PerCP

CD44-PE Cy 7

CD45-APC-H7

The antibody-labelled cells were then washed in PBS and resuspended before being analysed using a BD FACSCanto flow cytometer.

MSCs isolated by plastic adherence were used as a positive control in development of the algorithm. Briefly; these cells were isolated from bone marrow samples acquired from the AOC in the same process as outlined above. The donated bone marrow was broken up with a scalpel and washed in Hanks medium (Sigma H9269). The tissue (including bone, fat and cells) was then layered on Lymphoprep density gradient medium (Axis-Shield PoC AS) and centrifuged at 3000rpm for 30mins. The mononuclear cell layer was carefully removed and suspended in Dulbecco's modified eagle's medium (DMEM, D5523, Sigma). Red cells were subsequently removed by incubation in ammonium chloride lysis buffer (0.15M ammonium chloride, 0.01M potassium bicarbonate and 0.15mM EDTA in ddH2O) for 10 minutes at 4^{o}C and cell numbers calculated using trypan blue dye exclusion staining. The cells were then plated at $5x10^{3}cm^{-2}$ in DMEM supplemented with 10% foetal bovine serum (StemCell Technologies Inc., 06471) in vented culture flasks and passaged when reaching 80% confluence. Cells isolated in this way have previously been analysed in our laboratories and meet all of the agreed international criteria for identification of MSCs (surface markers and differentiation potential) as per ISCT standards.

At passage 3, cells were dehisced with trypsin EDTA (Cambrex, BE17-161E) and suspended in phosphate buffered saline at $2x10^{6}$ $cellsml^{-1}$. The cells were then incubated with 5µl of the same 7 fluorochrome-conjugated antibodies listed above, washed and resuspended in PBS supplemented with goat serum to prevent adherence to plastic flow cytometry counting tubes.

Identification of MSCs in peripheral blood was attempted using the same antibody combination as above. The samples were collected from patients attending the regional MS unit in accordance with Research Ethics Committee approval. Samples were collected in EDTA, stored at 4^{o}C and processed within 72 hours. Ammonium chloride lysis buffer was added to 2ml of whole blood and the resulting cells washed and then incubated with the same panel of 7 fluorochrome-conjugated antibodies. The sample was analysed using the same BD FACSCanto flow cytometer and gating strategy with multiparametric data analysed using Infinicyt^{TM} software.

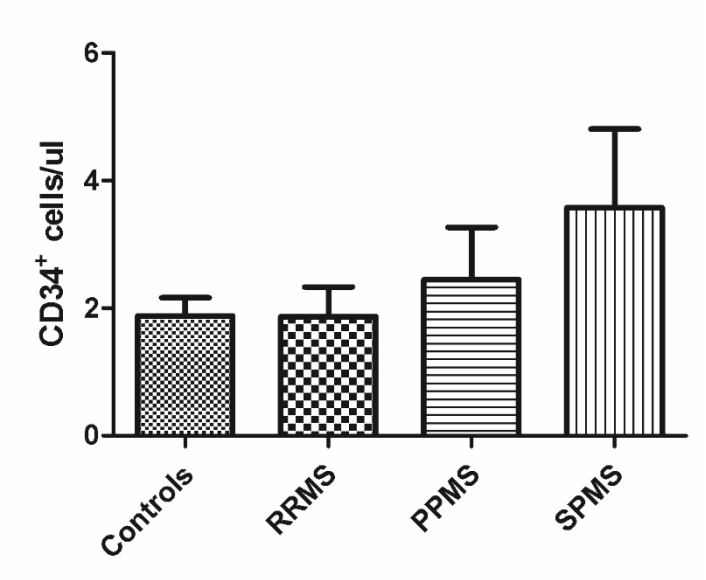
Results

Detection of haematopoietic stem cells;

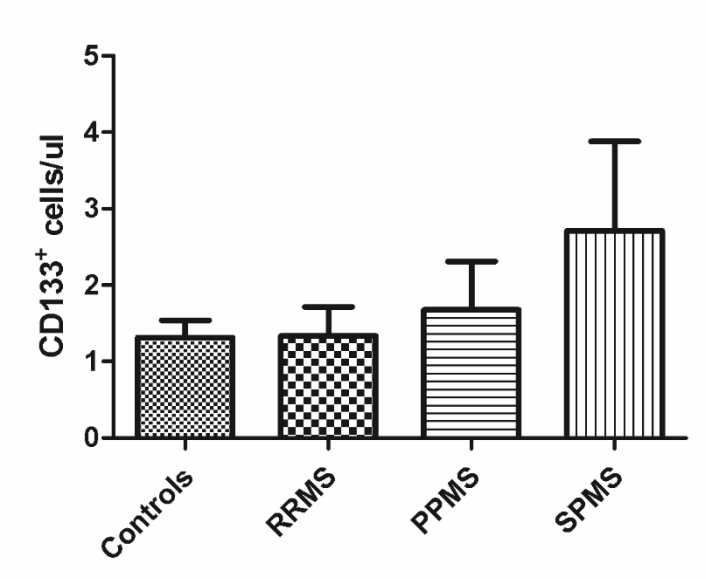
Blood samples were obtained from 6 controls and 67 patients with MS. Of those in remission and not on treatment, 7 had relapsing-remitting disease, 4 had primary progressive MS and 7 had secondary progressive disease. One-way analysis of variance showed that there was no difference between circulating numbers of $CD34^{+}$ cells in the blood of controls and the different subtypes of untreated MS *(Fig 3.2)* with an F-test value of 1.06 *(p=0.388)*, confirmed with Dunnett's post-hoc testing which showed there were no statistically significant differences between any of the disease subtypes and the control group.

Figure 3.2; A $CD34^+$ and **B** $CD133^+$ cell counts/µl in controls, RRMS, PPMS and SPMS. Error bars represent standard error of the mean (SEM).

A



B

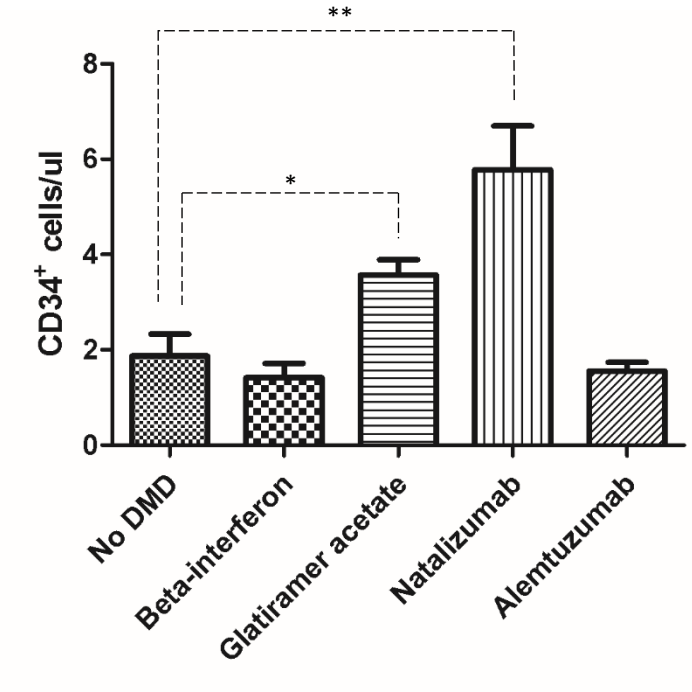


These results were replicated for $CD133^+$ cells for which the F-test value was 0.85 *(p=0.48).* Similarly there was no difference in the proportion of $CD34^+$ cells that were also positive for CD133 (data not shown) between the control group and the different types of MS (F=0.54; *p=0.66*).

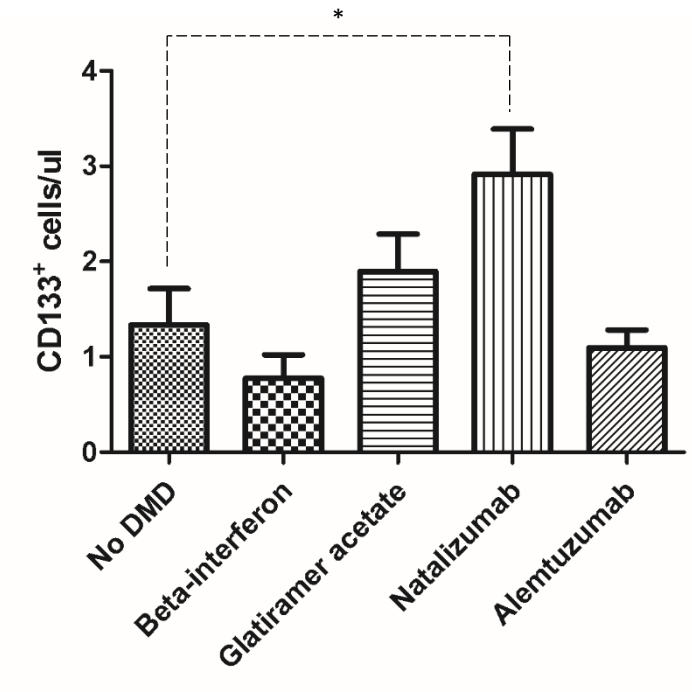
We next quantified the number of HSCs in the circulation of patients with RRMS taking four of the disease modifying therapies currently licensed in the UK (beta-interferon, glatiramer acetate, natalizumab and alemtuzumab). We first replicated work that has demonstrated mobilisation of $CD34^+$ HSC subsets in patients receiving anti-CD49d monoclonal antibody for the treatment of MS. Analysis of variance demonstrated statistically significant differences between groups (F=10.36; *p<0.*0001) and an unpaired students t-test a statistically significant difference between $CD34^+$ cell levels in patients not receiving DMD and those receiving monthly Natalizumab infusions (2-tailed unpaired t-test, *p=0.0046*). We also identified an increase in the numbers of circulating $CD34^+$ cells in those taking glatiramer acetate compared with those on no DMD (2-tailed unpaired t-test, *p=0.0197*). In multiparametric post-hoc testing (Dunnet's multiple conversion test) the only statistically significant difference was between the group taking no DMD and the group taking Natalizumab.

Fig 3.3; A $CD34^+$ and **B** $CD133^+$ cell counts/µl in patients with RRMS on no DMD, beta-interferon, glatiramer acetate, natalizumab and alemtuzumab (*p<0.05, **p<0.01)

A



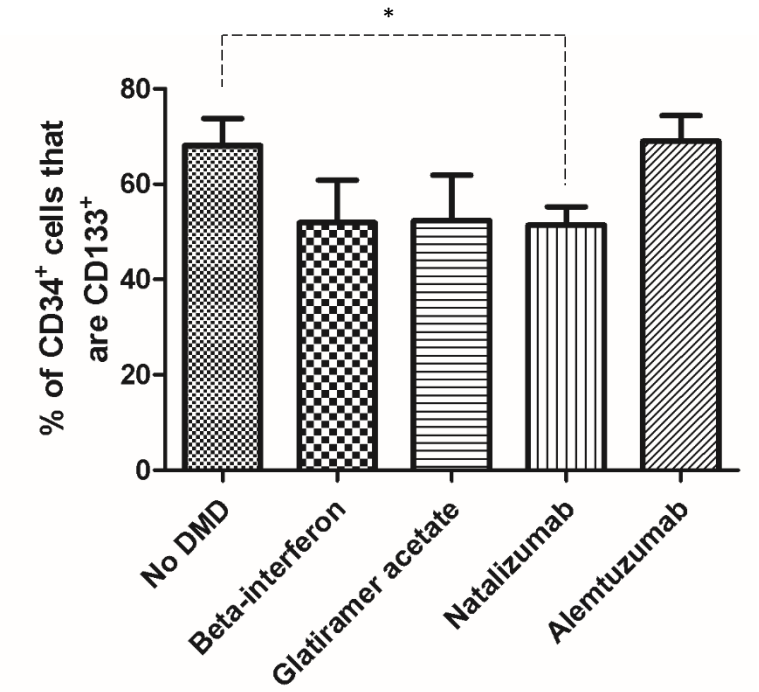
B



The results for circulating numbers of $CD133^+$ cells were similar though did not reach the same levels of statistical significance. Group analysis using one-way anova showed a difference between the group means (F=5.890, *p<0.0001*) with Dunnets showing statistically significant difference between the no DMD and Natalizumab groups. 2-tailed unpaired t-tests confirmed a statistical difference between the no DMD and Natalizumab groups (*p=0.0286*). There was no difference between the $CD133^+$ cell counts in the no DMD and Glatiramer groups (*p=0.3241*)

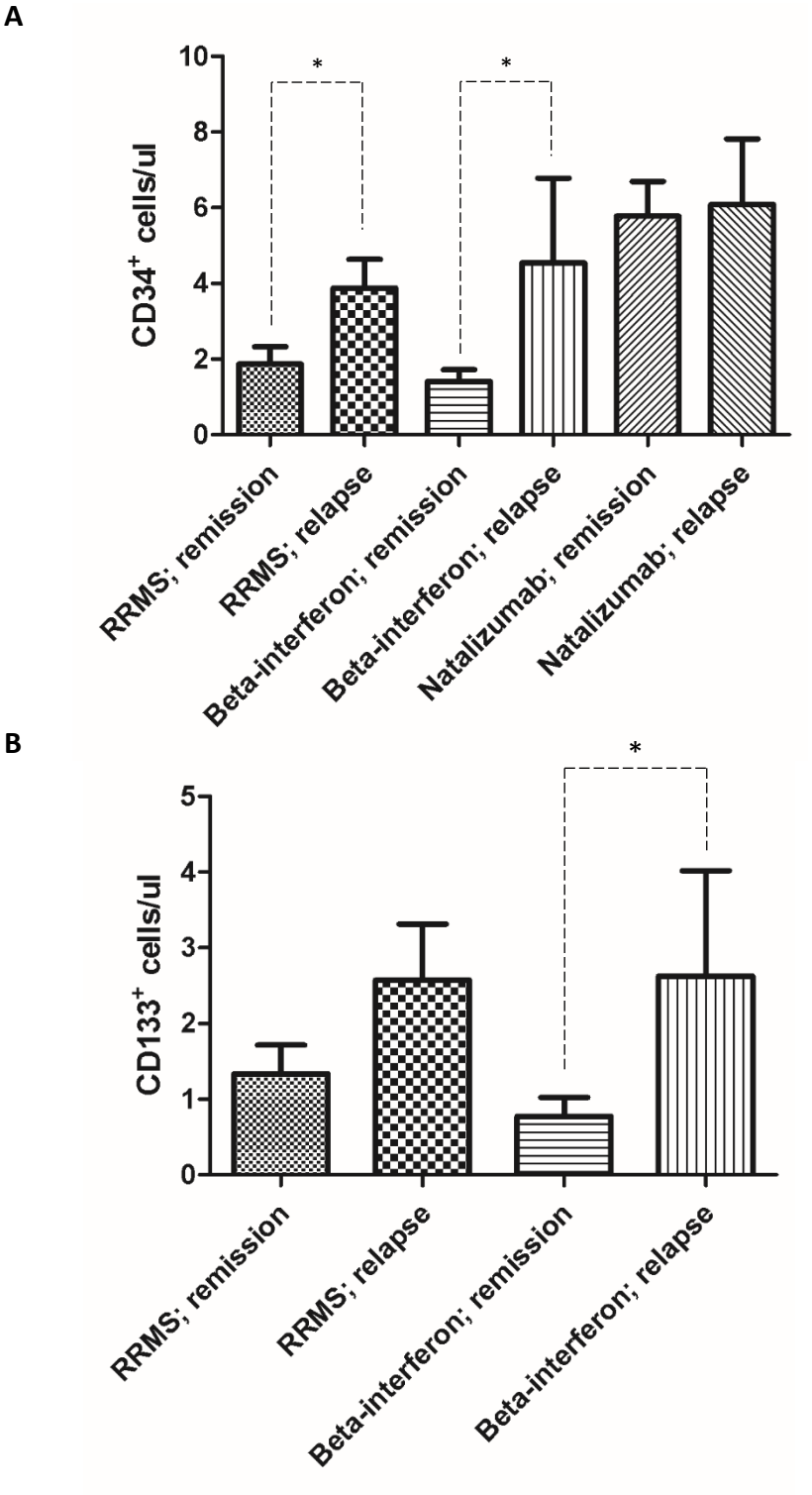
As has previously been described the proportion of $CD34^+$ cells that were also $CD133^+$ was reduced in those being treated with Natalizumab (unpaired t-test, *p=0.0221*). None of the other differences of group means reached statistical significance.

Fig 3.3; % of $CD34^+$ cells that are $CD133^+$ in patients with RRMS on no DMD, beta-interferon, glatiramer acetate, natalizumab and alemtuzumab (*p<0.05)



Finally we assessed circulating cell numbers in those undergoing clinically verified relapse warranting treatment with corticosteroid. Unpaired t-tests demonstrated a statistically significant increase in circulating $CD34^+$ cells in patients with RRMS undergoing relapse compared with those in remission regardless of whether they were receiving beta-inteferon (*p=0.0161*) or not (*p=0.0383*). A small increase was also demonstrated in those experiencing a relapse whilst receiving monthly Natalizumab infusions compared to those taking the same medications but in remission, though results were not statistically significant due to a small sample size of the relapse group. Unpaired t-tests also demonstrated a statistically significant difference in circulating numbers of $CD133^+$ cells in those undergoing relapse taking beta-interferon (*p=0.0363*). The same difference was not seen in those in the untreated groups.

Fig 3.4; A $CD34^+$ and **B** $CD133^+$ cell densities in patients experiencing relapse on no DMD, or taking beta-interferon or natalizumab (*p<0.05)



No difference was seen in the proportion of $CD34^+$ cells that were also positive for CD133 in patients undergoing relapse (data not shown).

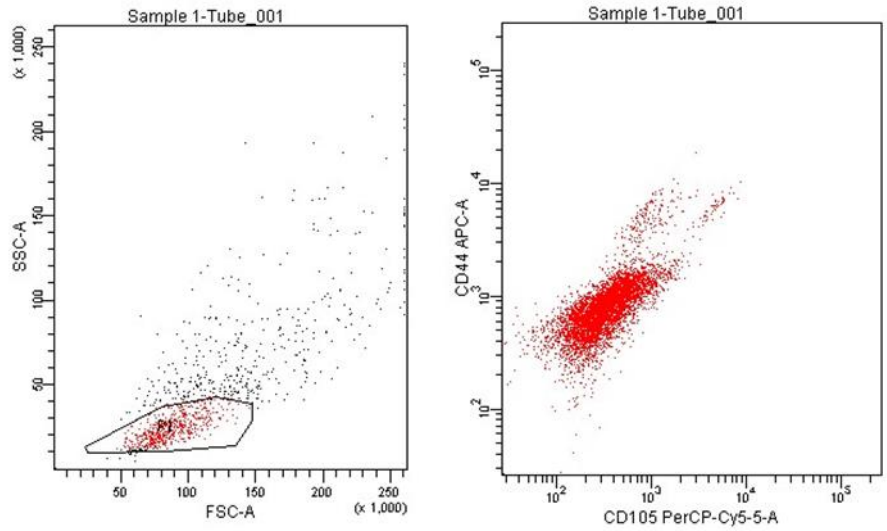
Detection of multipotent mesenchymal stromal cells;

Characterisation of cultured MSCs by flow cytometry

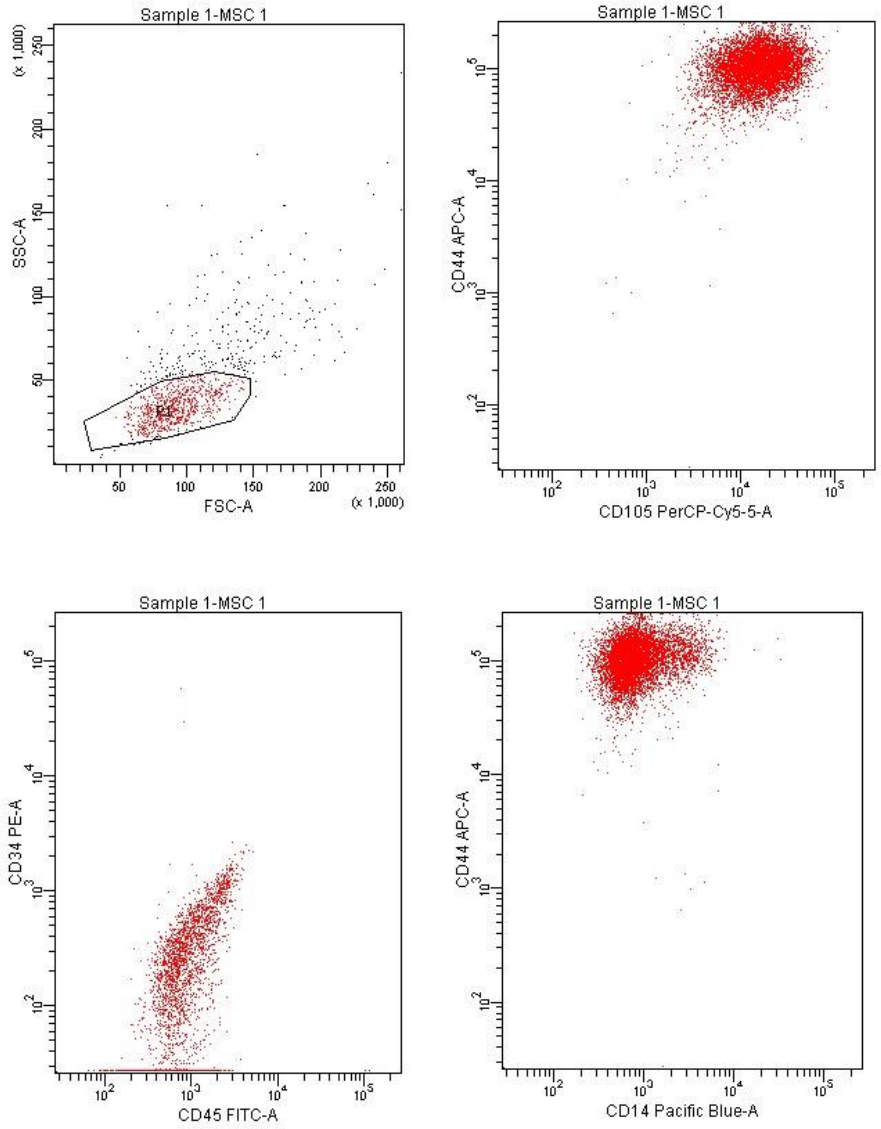
We first analysed laboratory-cultured MSCs - used as a positive control. The MSCs were isolated from the mononuclear cell fraction of whole bone marrow by plastic adherence in the conventional manner. The cells demonstrated homogeneous expression of CD44 and CD105 and were negative for expression of CD34 and CD45 (*Fig3.5b*).

Fig 3.5; Selected flow cytometry scatter plots of cultured MSCs; **A** isotype controls and **B** labelled with antibody panel as described

A



B

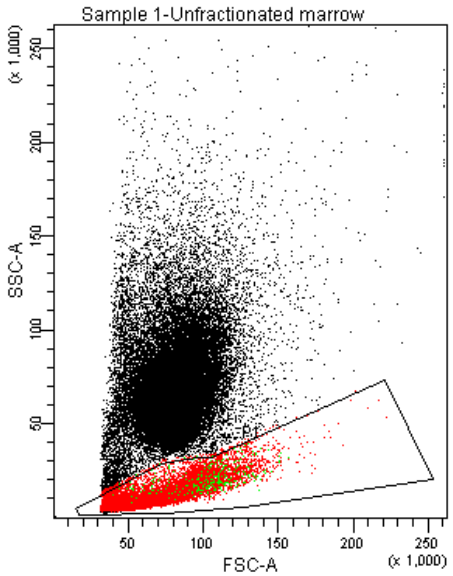

Sample 1-MSC 1
SSC-A
(x 1,000)
FSC-A
(x 1,000)
R1
Sample 1-MSC 1
CD44 APC-A
CD105 PerCP-Cy5-5-A
Sample 1-MSC 1
CD34 PE-A
CD45 FITC-A
Sample 1-MSC 1
CD44 APC-A
CD14 Pacific Blue-A

Identification of MSCs in whole bone marrow

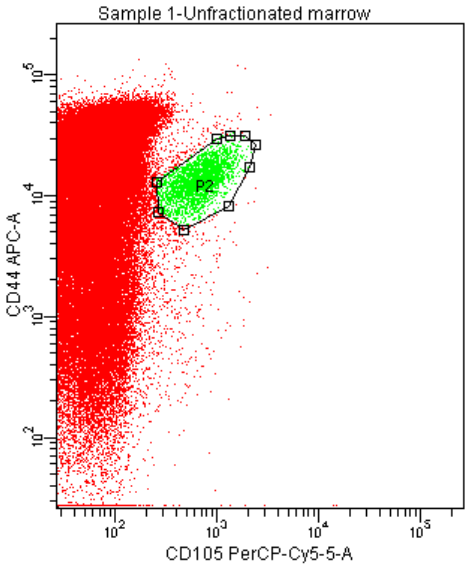
We next analysed whole bone marrow. The mononuclear population was gated according to forward scatter (cell size) and side scatter (cell granularity) in the conventional manner (*Fig 3.6a*). The flow cytometry scatter plot of CD44 and CD105 expression demonstrates the presence of the same homogeneous $CD44^{+}CD105^{+}$ cell population (represented in green) which is visibly distinct from the remaining $CD105^{-}$ mononuclear cells (*Fig 3.6b*). This population of cells are highlighted in green in *Fig 3.6c* in which they are demonstrably $CD34^{-}CD45^{-}$ in keeping with the phenotype of MSCs. Multiparametric analysis improves visual identification of the MSC population (*Fig 3.7*).

Fig 3.6; Flow cytometry scatter plots of whole bone marrow.

A



B


Sample 1-Unfractionated marrow
P2
CD44 APC-A
CD105 PerCP-Cy5-5-A

C

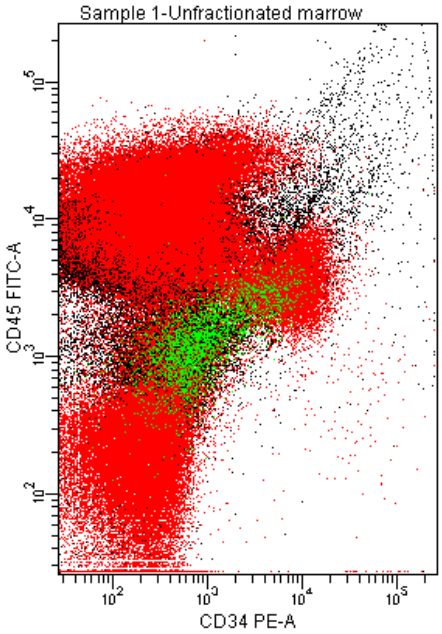
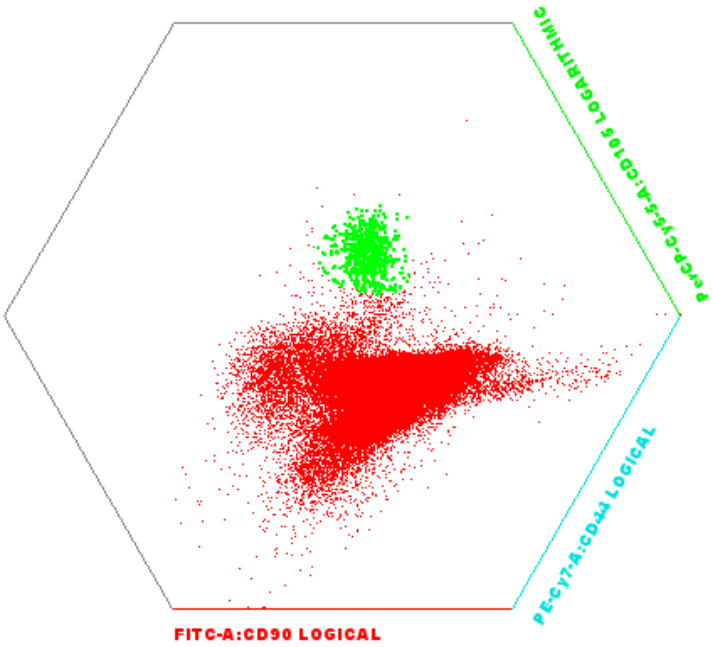

Sample 1-Unfractionated marrow
CD45 FITC-A
CD34 PE-A

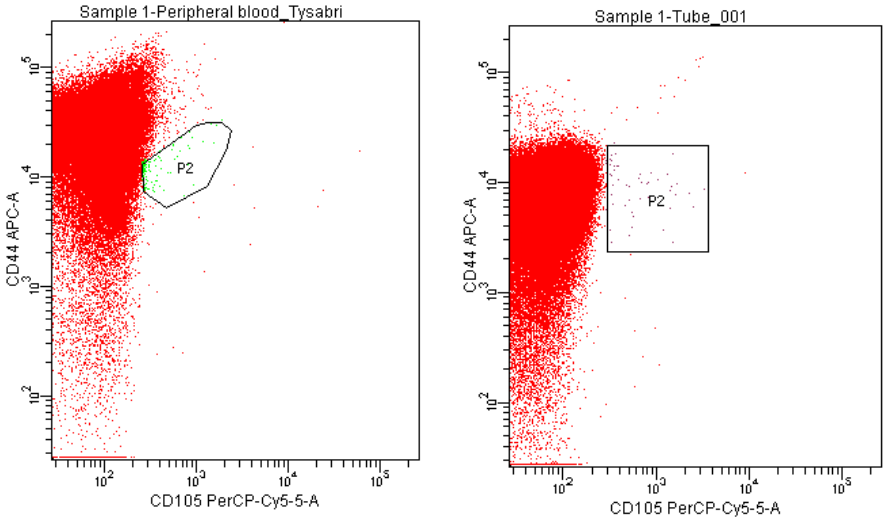
Fig 3.7; Use of multiparametric scatter plots enables clearer visual separation of MSCs (green) from alternative MNCs (red)



Identification of MSCs in blood samples from patients with MS

We next attempted to identify the same population of cells in the peripheral blood samples of patients with MS including in those with 'mobilised' blood who were taking anti-CD49d antibody. In Fig 3.7 there are two examples of flow cytometry scatter plots of peripheral blood samples from patients receiving Natalizumab. The same population of $CD44^{+}CD105^{+}$ cells were not identifiable in the samples.

Fig 3.7; Flow cytometry scatter plots of blood samples obtained from patients taking Natalizumab for the treatment of RRMS – the same population of $CD44^{+}CD105^{+}$ cells are not identifiable.



Discussion

We have successfully demonstrated mobilisation of HSCs into the peripheral blood during treatment with Natalizumab consistent with previously published work (Zohren et al., 2008). Natalizumab is an antibody to the α4 subunit of α4β1 integrin, a cell adhesion molecule which through avidity to vascular cell adhesion molecule-1 plays a key role in leucocyte trafficking from the peripheral blood into various components of the extracellular matrix, including to the CNS. The clinical relevance of this mobilisation is not fully understood. Recent work has shown that these cells retain full expansion and differentiation capacity, are more quiescent and are associated with differential effects on the frequency of circulating naive and memory B cells and of regulatory T cells which may explain correlation with amelioration of disease activity (clinical and radiological) (Mattoscio et al., 2015).

We have also identified a similar elevation of circulating cell types in treatment with Glatiramer acetate which is a novel finding. A putative mechanism for this effect is more difficult to envisage given the lack of biological activity of a co-polymer of 4 amino acids which is thought to have its principal effect through immune system decoy. Further interrogation is required to establish whether these cells have a meaningful clinical effect.

We have also shown spontaneous mobilisation of HSCs into the circulation during a clinical relapse of MS, again with potential physiological relevance with regard to the endogenous inflammatory response. The $CD133^+$ cell subset have already been shown to possess neuroprotective properties in the context of other neurological disease (Bakondi et al., 2009) and to promote reparative potential through enhancement of progenitor cell survival (Bakondi et al., 2011). Their presence in this context may therefore contribute both in attenuating tissue injury and promotion of endogenous repair mechanisms.

We have also developed a novel means of detecting MSCs in the peripheral blood, although we did not detect peripheral blood mobilisation of MSCs under any of the conditions tested in either MS patients or control subjects. Rare event detection in flow cytometry remains challenging. The use of manual techniques including gating and visual identification of cell populations has associated challenges (Lugli et al., 2010) and the volumes of non-mobilised peripheral blood required to isolate MSCs gives evidence of the rarity of the cells we are attempting to identify (Zvaifler et al., 2000). The ability to track stem cell numbers, phenotype and differentiation remains a key goal to understanding some of the effects of intravenous administration of stem cell populations in ours and other work. Advances in flow cytometry technology including the development of mass spectrophotometry-based cytometers (Bendall et al., 2012) and the ability to process complicated multiparametric data with automated computational methods may enable

confident and reproducible progress in reaching this goal in the future (Aghaeepour et al., 2013).

Our results suggest a role for HSC mobilisation during treatment of MS and in acute relapse. Further work will be required to definitively determine whether mobilisation of endogenous stem cells correlates with recovery and is reparative or protective against future progression. The mechanisms underlying the putative contribution to repair also remain to be definitvely established.

Chapter 4

Quantification of neurodegeneration in MS subjects

Introduction

Following publication of the first phase III therapeutic trials in Multiple Sclerosis (MS) and their subsequent regulatory approval in the 1990s, attention turned to the matter of robust outcome measures to try and ensure reliable identification of therapeutic effect. 20 years later meaningful progress remains elusive and the design and validation of disability outcome measures continues to be a key requirement for all phenotypes of multiple sclerosis. Within the primary progressive MS (PPMS) cohort this is particularly pertinent given an absence of clinical relapses or prominent MRI inflammatory activity, and provides significant barriers to robust therapeutic studies.

The expanded disability status scale (EDSS), a 10-point ordinal scale based on neurological assessment and ambulation, was designed in the 1960s by John Kurtzke and modified 20 years later (KURTZKE, 1961; Kurtzke, 1983). It is familiar (due to the accumulated decades of use) and, perhaps most crucially, is accepted by regulators as a measure of disease progression. It does however have significant

shortcomings, widely discussed in the literature (Hobart et al., 2000). It is an ordinal scale with significant functional intervals in its higher reaches, making progression partly a function of baseline score (Weinshenker et al., 1991). Perhaps the most telling criticism is its inadequate sensitivity and responsiveness to changes in disease status (Whitaker et al., 1995). In the context of PPMS it is insufficiently sensitive to enable detection of therapeutic effect in manageable cohort numbers over trial-length timeframes (Cottrell et al., 1999). Despite its widely recognised shortcomings, its historical use, particularly in the context of the large (untreated) natural history studies which are no longer ethically possible, means it is probably indispensable in the context of design of updated outcome measures or surrogates. Alternative clinical scoring systems such as the MSFC have been developed due to these recognised shortcomings (Cutter et al., 1999; Polman and Rudick, 2010) but despite proven correlation with clinical (Rudick et al., 2009), imaging (Kalkers et al., 2001) and patient-reported outcomes (Miller et al., 2000), it has not been acceptable to regulators as a primary endpoint in therapeutic studies, principally due to its dimensionless output.

Alternatives to disability scales include composite endpoints such as disease activity free status, global measures of activities of daily living (such as Rankin scale/Barthel index), patient reported outcomes and other novel techniques such as kinematic recording. None of these have thus far been validated. Biomarkers are an attractive alternative if they are quantitative, reproducible and sensitive to small changes in disease status. Whilst conventional MRI sequences have been validated

as a trial-level surrogate endpoint for relapse in RRMS (Sormani et al., 2009), they have not for disability progression or reversal.

Neurophysiological assessment in the form of multimodal evoked potentials fulfil many of the criteria of an effective biomarker, and have been shown to have clear correlation with clinical disability in RR and SPMS in cross-sectional (Invernizzi et al., 2011; Leocani et al., 2006) and longitudinal studies (Fuhr et al., 2001; Jung et al., 2008; Schlaeger et al., 2012b). A small study with not insignificant participant drop-out has been published in the PPMS cohort and found similar correlations. This work did not include direct comparison with imaging metrics (Schlaeger et al., 2014a). Of most interest from an imaging perspective are measures of axonal integrity/loss thought to underscore progressive disability; these include grey matter atrophy, cord atrophy, diffusion tensor imaging and magnetisation transfer ratio.

The PPMS patient population provide a cohort that are unencumbered by clinical relapses, to some representing an ideal substrate in the search for alternative biomarkers. A longitudinal study of evoked potential parameters in the PPMS cohort was conceived and initiated through the Neurology department at Frenchay Hospital in 2008. Analysis of these data is here performed in a cross-sectional and longitudinal format. Additionally, 27 of the 29 participants agreed to attend for MRI imaging (analysed by collaborators at the University of Nottingham) and repeat

clinical scoring to enable within-subject comparison of imaging and neuronal function (through evoked potential analysis).

Materials and Methods

Neurophysiological data was collected from 29 patients recruited from a regional MS centre. Inclusion criteria included a definite diagnosis of PPMS according to the revised McDonald criteria of 2005 (Polman et al., 2005) and written informed consent. Exclusion criteria included contraindications to transcranial magnetic stimulation and concomitant neurological disease. The study was approved by the local ethics committee.

Neurophysiological investigations were carried out at 0, 6, 12, 24, and 36 months and consisted of visual EPs (VEPs), motor EPs (MEPs) to adductor pollicis brevis and tibialis anterior, median and tibial somatosensory EPs (SSEPs) and brain stem auditory EPs (BSAEPs). At each visit the subject also underwent clinical assessment by a Neurologist or MS specialty doctor; this consisted of the Expanded Disability Status Scale (EDSS) and the MS Functional Composite (MSFC). The participants also completed the Rivermead Mobility Index (RMI) questionnaire. 27 of the

participants underwent single time-point MRI imaging and repeat clinical assessment consisting of the EDSS, MSFC and RMI.

Data were analysed on a cross-sectional and longitudinal basis. Comparison of the disability-correlation of neurophysiological and imaging parameters was completed in the 27 patients who consented to undergo MRI imaging. The most recent complete neurophysiological dataset was used from each participant. At the time of Neurophysiological assessment, the mean age of the participants was 52.4 years with a mean disease duration of 131.9 months. At the time of MRI imaging the mean age was 54.3 years with a mean disease duration of 154.0 months. The mean time lapse between Neurophysiological recording and MRI imaging was 671 days with a minimum of 254 days and a maximum of 1492 days. There were 14 male participants and 13 female.

For the longitudinal analysis, data were available from 27 patients who completed multiple visits. Of the 29 patients recruited to the study, data was available for the purposes of this thesis from; 15 who completed all 5 visits/3 years of the study, 7 who completed 4 visits/2 years, and 5 who completed 3 visits/1 year of the study. The mean age of participants at baseline was 50.1 years with a mean disease duration of 102.2 months. Data were acquired from 13 female and 14 male patients.

Evoked potentials – acquisition;

All EPs were recorded using a Synergy Medelec machine according to International Federation of Clinical Neurophysiology (IFCN) guidelines (Deuschl and Eisen, 1999). Reference ranges were taken from the IFCN datasets except for VEPs in which reference ranges were calculated using mean and standard deviation values taken from internal laboratory recordings. In brief;

- VEPs were recorded 5cm anterior to the inion (Oz) with a reference electrode at Fz. Full field pattern-reversal stimulation was presented to each eye at a frequency of 2 Hz and luminance of 85 candela/m^2. 100 sweeps were averaged and the p100 latency was identified from the resulting montage.
- BSAEPs were recorded at Cz with the reference electrode placed over the ipsilateral mastoid. Rarefaction clicks at 70 dB above hearing threshold were delivered at 10.1 Hz and 2000 sweeps averaged to allow identification of the waveforms III and V and calculation III-V.
- SSEPs were recorded in both the upper and lower limbs. For median nerve SEPs the recording electrodes were placed at Cv (over the 5^{th} cervical spinous process) and Cc (2cm posterior to Cz and 7cm contra-lateral to the midline) with the reference electrodes positioned at antCv (over the supra-glottal region) and Fz. For tibial SEPs the recording electrodes were positioned at Lum (over the L2 spinous process) and Cc (as above) with reference electrodes over the umbilicus and at Fz. Stimulation was performed at motor threshold with a 0.2ms square wave pulse at 3 Hz. 1000

sweeps were recorded for each potential and major components (p37 and lum for the posterior tibial SSEP (p37-lum) and N13 and N20 for median nerve SSEPs (N13-N20)) recorded from the resulting montage.

- MEPs were generated using a Magstim unit with a 9cm circular stimulating coil. Recording was made at the target muscles (adductor pollicis brevis in upper limbs; abductor hallucis in lower limb) and stimulation repeated until 10 50-100uV responses had been recorded. M- and F-waves were recorded for median (16 trials) and posterior tibial (10 trials) nerves and CMCT calculated using the formula; CMCT = MEP latency - (F-wave latency + M-wave latency – 1)/2 where the M-wave is the distal motor latency.

Evoked potentials – scoring;

Conversion of the raw latency and amplitude data acquired during neurophysiological testing was based on a series of techniques that have previously been described in the literature. Lower limb latency limits were adjusted for height as per Jung et al (Jung et al., 2008).

The first was a 'conventional' ordinal scoring system modified from Fututake et al (Fukutake et al., 1998) by Leocani and colleagues (Leocani et al., 2006) that grades each evoked potential according to a 4 point scale;

0 Normal

1 Increased latency

2 Increased latency + morphological abnormality of a major component

3 Absence of a major component

Each potential was analysed independently by 2 Neurophysiologists and summated to give a score between 0 and 36 (bilateral VEP, BSAEP, UL SSEP, UL MEP, LL SSEP and LLMEP each scored 0-3).

The second was a 6 point ordinal scale based solely on latency as described by Jung et al (Jung et al., 2008);

0 Normal

1 Pathological side difference of latency

2 Latency of upper limit of normal (mean + 2.5 standard deviations) (ULN) to 1.1 x ULN or >50% side difference of amplitude

3 Latency of 1.1-1.3 x ULN

4 Latency >1.3 x ULN

5 Absent EP component

Each potential was scored and summated to give a score of 0 - 60 (2 sides, 6 EPs, 5 points per EP).

The data was also converted to z-scores for each EP whereby;

z = (actual latency – mean control latency) / control standard deviation

The z-scores were then summed (the **sum-z score**). The use of z-scores is complicated by absent EP components. Various techniques have been employed

to circumvent this problem. In order to preserve the relevance of an absent component and to reflect greater pathway dysfunction we substituted absent EPs with the cohort max z-score + 1.

Finally, the number of abnormal EPs divided by the number of recorded EPs was calculated yielding the **Path-Q** score as performed by Schlaeger and colleagues (Schlaeger et al., 2014a)

MR Imaging;

All consenting participants were imaged using a Phillips 3T MR scanner. Brain imaging was recorded using a 32 channel coil and $1mm^3$ voxels. Sagittal 3D T1-weighted fast gradient echo sequences were used for volumetric analysis and fluid attenuation inversion recovery for white matter lesion quantification. Spinal imaging was performed using a Sense Spine Coil. Cross-sectional areas at 4 spinal levels were calculated from T2 balanced gradient echo sequences using contrast level appearance.

All MRI parameters were calculated by collaborators at the University of Nottingham who were supplied with copies of the raw data in anonymised format. Details of their methods are not available for the purposes of this thesis.

Statistical analysis;

Cross-sectional and longitudinal correlations were calculated using Spearman's correlation coefficient (*rho/ρ*) with consensus statistical significance set at p values of 0.05 and 0.01. Data was analysed using Prism software. Where multiple values were taken from the same participant multilevel statistical modelling was employed using Stata statistical software.

Results

Cross sectional analysis; neurophysiology

Correlation of Neurophysiology-derived global scores of CNS dysfunction with clinical disability metrics revealed strong correlation consistent with previous work in MS populations. Of the global disability metrics, EDSS better correlates with the various methods of EP analysis (rho/*ρ* = 0.65- (-)0.71) than does the MSFC (rho/*ρ* = 0.47- (-)0.50) *(Fig 4.1).* Elements that reflect/quantify mobility including the RMI and ambulatory threshold (a component of the EDSS), both showed some statistically significant correlation with the EP scores – this is unsurprising given that the EDSS is heavily weighted towards mobility in the upper echelons of the scale. Perhaps surprisingly, whilst there was no correlation of the neurophysiological scores with disease duration, there was some correlation with age of the patient

(Table 4.1). Of the different evoked potential scoring methods, that based on z-transformed latencies showed the strongest correlation with the EDSS compared with ordinal scales (rho = -0.71 vs 0.65-67 with ordinal scoring methods - all p values < 0.05).

Figure 4.1; Scatter plots of the EDSS versus the 4 different neurophysiology-based scoring methods; **A** ordinal 4-point score (rho = 0.65, p = 0.0003); **B** ordinal 6-point score (rho = 0.67, p = 0.002); **C** Summed z-score (rho = -0.71, p < 0.0001); **D** Path-Q score (rho = 0.67, p = 0.0002). Each dot represents 1 of the participants. Line of best fit calculated using multiple regression analysis.

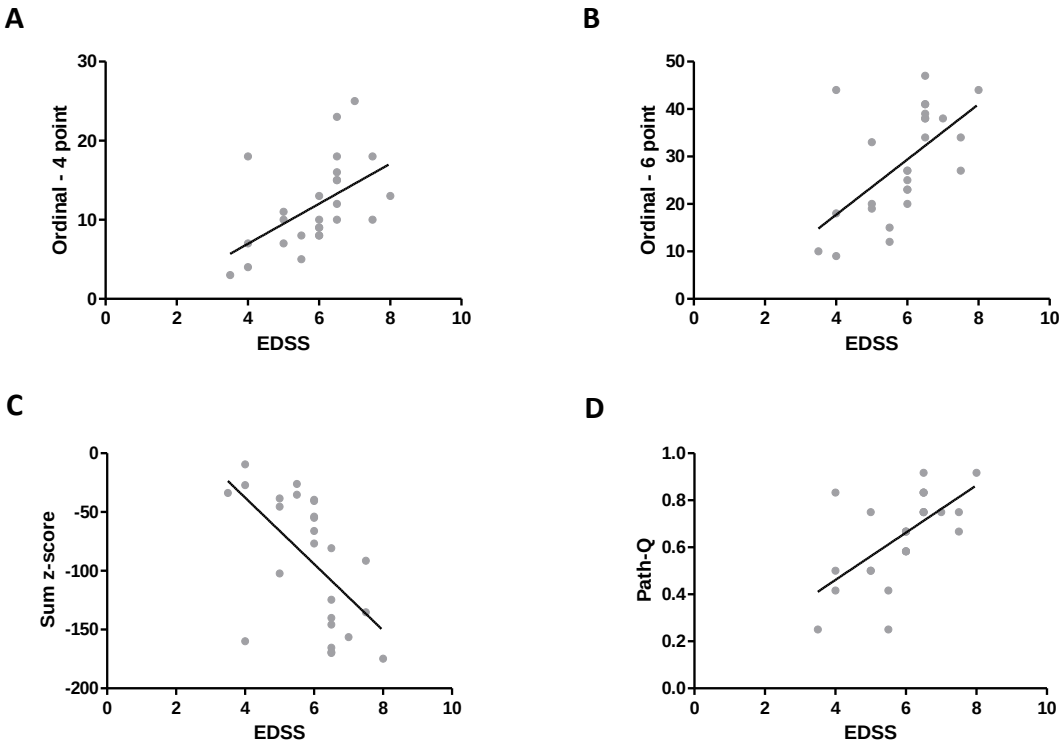


Table 4.1; Spearman's correlation coefficients (rho/ρ) of the four different neurophysiological scoring methods and patient demographics, EDSS, ambulation (distance covered within EDSS assessment), MSFC and Rivermead Mobility Index (RMI). P-values of 0.05 or less are indicated in bold red, values of 0.01-0.05 are indicated in red.

	Ordinal - 4 point		Ordinal - 6 point		Summed z-score		Path-Q	
	rho	p	rho	p	rho	p	rho	p
Age	**-0.53**	**0.005**	**-0.52**	**0.006**	0.50	0.0101	-0.47	0.014
Disease duration	0.21	0.31	0.10	0.61	-0.10	0.63	0.00	1.00
EDSS	**0.65**	**0.0003**	**0.67**	**0.0002**	**-0.71**	**<0.0001**	**0.67**	**0.0002**
Ambulation	**-0.55**	**0.003**	**-0.56**	**0.003**	**0.64**	**0.001**	**-0.54**	**0.005**
MSFC	**-0.50**	**0.009**	-0.46	0.02	0.47	0.015	-0.49	0.011
RMI	**-0.55**	**0.003**	**-0.56**	**0.003**	**0.60**	**0.001**	**-0.59**	**0.001**

The pathways interrogated in each of the evoked potentials are differentially affected both between and within subjects. Some of the evoked potentials (such as MEP and VEP) have been shown to have greater change over time and better correlation with disease metrics (Fuhr et al., 2001). Correlations of the component parts of the global evoked potential scores and varying combinations thereof with the clinical metrics were next calculated *(Table 4.2).*

In this dataset, the performance of individual evoked potentials as correlates of clinical scores of disease severity depended on the scoring method employed. Using a 4-point ordinal scale (calculated according to amplitude/morphology and latency), the MEP correlated best with the EDSS (rho=0.59, p=0.002) though this was less than when using a global score (derived from all 4 evoked potentials). When using a more sensitive 6-point scale based only on latency (rho=0.75, p<0.0001) or z-transformed latencies (rho=0.72, p<0.0001), combined upper and lower limb SSEPs provided the best correlation with EDSS and this was superior to the performance of global scores.

Using the 4-point ordinal scale or the z-transformed latencies, exclusion of evoked potentials from the global score did not improve on performance. However when using a 6-point ordinal scale based on latency, combining the VEP score with the lower limb MEP and both upper and lower limb SSEP improved correlation with the

EDSS (excluding the BSAEP and upper limb MEP) compared with the global score (rho=0.78, $p<0.0001$).

Correlation of individual evoked potential scores with the MSFC was improved by excluding the PASAT from the clinical score but, as with global evoked potential scores, correlation was less than with the EDSS.

Table 4.2; Spearman's correlation coefficients (rho/ρ) of clinical measures (including selected sub-components) and evoked potential scores (calculated according to ordinal scores and z-transformed latencies as previously described) either individually or in various combinations.

The clinical scores include the average of the 9HPT and T25FW (MSFC without paced serial addition test).

The evoked potentials used in the analysis consist of motor-evoked potentials (MEP) and somatosensory evoked potentials (SSEP) including those within the upper limbs only (UL) and lower limbs only (LL), and the visual evoked potentials (VEP), individually and in various combinations.

P-values of 0.05 or less are indicated in bold red, values of 0.01-0.05 are indicated in red.

	EDSS		Ambulation		MSFC		9HPT		T25FW		9HPT/T25FW	
	rho	p	Rho	p	rho	p	rho	p	rho	p	rho	p
4-point ordinal scale; latency and morphology												
MEP	**0.5887**	**0.0016**	**-0.53**	**0.0054**	-0.394	0.0465	-0.324	0.1058	-0.320	0.11	-0.3585	0.0721
SSEP	0.4766	0.0138	-0.379	0.0561	-0.427	0.0298	-0.448	0.0215	-0.261	0.1972	-0.4666	0.0163
SSEPLL	0.3343	0.0951	-0.204	0.3167	-0.188	0.3584			-0.073	0.7219	-0.1692	0.4086
VEP	0.3654	0.0664			-0.094	0.648						
5-point ordinal scale; latency only												
MEP	**0.5072**	**0.0082**	-0.371	0.0623	-0.271	0.1806	-0.292	0.1479	-0.275	0.1748	-0.3183	0.113
MEPUL	0.434	0.0267			-0.221	0.2771	-0.288	0.1538			-0.2629	0.1945
MEPLL	**0.592**	**0.0014**	**-0.514**	**0.0072**	-0.416	0.0347			-0.283	0.1616	-0.3932	0.0469
SSEP	**0.7492**	**<0.0001**	**-0.729**	**<0.0001**	**-0.534**	**0.0049**	**-0.558**	**0.0031**	**-0.510**	**0.0078**	**-0.606**	**0.001**
SSEPUL	**0.5109**	**0.0077**			-0.414	0.0356	**-0.553**	**0.0034**			**-0.5471**	**0.0038**
SSEPLL	**0.5097**	**0.0078**	**-0.501**	**0.0092**	-0.378	0.0566			-0.306	0.1281	-0.3015	0.1344
VEP	0.3162	0.1155			-0.217	0.2866						
MEPLL/SSEP	**0.7733**	**<0.0001**	**-0.72**	**<0.0001**	**-0.535**	**0.0049**	**-0.535**	**0.0049**	-0.489	0.0112	**-0.5906**	**0.0015**
MEPLL/SSEP/VEP	**0.7799**	**<0.0001**	**-0.704**	**<0.0001**	**-0.564**	**0.0027**	**-0.532**	**0.0051**	**-0.502**	**0.0089**	**-0.5965**	**0.0013**
z-transformed latencies												
MEP	**-0.654**	**0.0003**	**0.5287**	**0.0055**	0.427	0.0296	0.3696	0.0631	0.3237	0.1067	0.401	0.0423
MEPUL	**-0.552**	**0.0035**			0.3121	0.1206	0.3566	0.0738			0.3703	0.0626
MEPLL	**-0.637**	**0.0005**	**0.5547**	**0.0033**	0.4209	0.0322			0.3056	0.129	0.3859	0.0515
SSEP	**-0.715**	**<0.0001**	**0.731**	**<0.0001**	**0.5439**	**0.0041**	**0.5829**	**0.0018**	**0.4992**	**0.0094**	**0.6342**	**0.0005**
SSEPUL	**-0.54**	**0.0044**			0.417	0.034	**0.5819**	**0.0018**			**0.5806**	**0.0019**
SSEPLL	**-0.511**	**0.0076**	**0.4989**	**0.0095**	0.3537	0.0763			0.2957	0.1424	0.2773	0.1702
VEP	-0.296	0.1423			0.2072	0.3098						

Cross-sectional analysis; imaging

In cross-sectional analysis of imaging parameters, only spinal cord area correlated to any degree with clinical motor disability measures *(Table 4.3)*. The C5/6 cross sectional area (CSA) was the only measure to correlate with the EDSS (rho=0.54, p=0.02), as well as the only to correlate with mobility threshold, MSFC and 9HPT/T25FW. In contrast to the evoked potential scores, correlation was better with the MSFC (rho=0.63, p=0.006). Whilst the C2/3 CSA correlated well with the 9HPT (rho=-0.73, p=0.003), none of the imaging parameters correlated with the T25FW.

The only objective clinical score of cognitive function tested in this study was the PASAT. Volumetric imaging parameters showed a trend towards correlation with the PASAT though only one, white matter volume, reached statistical significance (rho=0.49, p=0.03).

Table 4.3; Table of Spearman correlation coefficients (ρ/rho) of clinical measures (including selected sub-components) and imaging parameters. Clinical parameters as in table 4.2 with the addition of the paced serial addition test. Abbreviations; EDSS = expanded disability status scale; MSFC = multiple sclerosis functional composite; 9HPT = 9-hole peg test; T25W = timed 25 foot walk; PASAT = paced serial addition test; GMV = grey matter volume; WMV = white matter volume; BV = brain volume; TIV = total intracranial volume; CSA = cross-sectional area. P-values of 0.05 or less are indicated in bold red, values of 0.01-0.05 are indicated in red.

	EDSS		Ambulation		MSFC		9HPT		T25FW		9HPT/T25FW		PASAT	
	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p
GMV (litres)	0.05	0.83	0.02	0.93	0.05	0.85	-0.09	0.72	-0.19	0.43	-0.17	0.48	0.43	0.07
WMV (litres)	-0.01	0.98	0.08	0.74	0.12	0.62	-0.12	0.63	-0.18	0.46	-0.12	0.63	0.49	0.03
BV (litres)	0.05	0.85	0.02	0.94	0.05	0.85	-0.08	0.74	-0.17	0.50	-0.18	0.47	0.45	0.06
BV/TIV	-0.12	0.64	0.23	0.34	0.27	0.27	-0.05	0.84	-0.07	0.78	0.08	0.76	0.42	0.07
Lesion Load (litres)	0.28	0.25	-0.34	0.16	-0.27	0.26	0.20	0.41	0.25	0.31	-0.25	0.30	-0.18	0.47
Lesion Load (%)	0.26	0.29	-0.33	0.16	-0.30	0.21	0.22	0.37	0.24	0.33	-0.24	0.31	-0.29	0.23
C2/3 CSA (mm^2)	-0.41	0.14	0.48	0.09	0.53	0.054	**-0.73**	**0.003**	-0.30	0.29	0.49	0.08		
C5/6 CSA (mm^2)	-0.54	0.02	0.58	0.015	**0.63**	**0.006**	-0.52	0.03	-0.15	0.56	0.51	0.04		
T4/5 CSA (mm^2)	-0.35	0.16	0.30	0.23	0.42	0.09	-0.47	0.049	-0.10	0.68	0.28	0.26		
T9/10 CSA (mm^2)	-0.29	0.25	0.28	0.26	0.35	0.15	-0.35	0.15	0.00	0.99	0.20	0.43		

Post hoc analysis with comparison of median-split groups according to age and disease duration was undertaken, demonstrating greater correlation of EDSS and EP score in older participants and those with longer disease duration for the majority of the scoring techniques *(Tables 4.4 and 4.5)*. Indeed, restricting calculations to include only those in the oldest 50% of the cohort and the 50% with the longest disease duration improved correlations (across all EP scoring methods) compared with calculations using the entire dataset.

The EP scores demonstrated more sporadic correlation with the MSFC and to a lesser degree than with the EDSS in all except one of the statistically significant results.

Table 4.4; Spearman's correlation coefficients (ρ/rho) for median-split subgroups (according to disease duration) of EDSS and MSFC and the neurophysiological scores as previously described. P-values of 0.05 or less are indicated in bold red, values of 0.01-0.05 are indicated in red.

	EDSS				MSFC			
	rho	p	rho	p	rho	p	rho	p
	Lower half		Upper half		Lower half		Upper half	
4-point ordinal scale; latency and morphology								
Global score	0.4548	0.1184	**0.8669**	**0.0001**	-0.4288	0.1438	-0.4814	0.0958
5-point ordinal scale; latency only								
Global score	0.4881	0.0906	**0.8396**	**0.0003**	0.449	0.1237	-0.4077	0.1667
MEPLL/SEP	**0.709**	**0.0067**	**0.8211**	**0.0006**	-0.5069	0.0771	-0.4545	0.1186
MEPLL/SEP/VEP	0.6167	0.0248	**0.8994**	**<0.0001**	-0.5007	0.0814	-0.5482	0.0524
Sum of z-transformed latencies								
Global score	-0.5583	0.0474	**-0.8118**	**0.0008**	0.467	0.1076	0.3846	0.1944
SEP	**-0.8052**	**0.0009**	-0.6017	0.0296	0.6484	0.0165	0.2473	0.4154
Path Q	0.4908	0.0886	**0.8527**	**0.0002**	-0.4318	0.1406	-0.5465	0.0533

Table 4.5; Spearman's correlation coefficients (ρ/rho) for median-split subgroups (according to age of patient) of EDSS and MSFC and the neurophysiological scores as previously described. P-values of 0.05 or less are indicated in bold red, values of 0.01-0.05 are indicated in red.

	EDSS				MSFC			
	rho	p	rho	p	rho	p	Rho	p
	Lower half		Upper half		Lower half		Upper half	
4-point ordinal scale; latency and morphology								
Global score	0.3437	0.2501	**0.9382**	**<0.0001**	-0.3126	0.2984	-0.5317	0.0615
5-point ordinal scale; latency only								
Global score	0.2348	0.44	**0.9495**	**<0.0001**	-0.1909	0.5322	-0.6253	0.0223
MEPLL/SEP	0.6179	0.0244	**0.903**	**<0.0001**	-0.3807	0.1994	-0.4821	0.0952
MEPLL/SEP/VEP	0.5318	0.0614	**0.931**	**<0.0001**	-0.3576	0.2302	-0.6704	0.0122
Sum of z-transformed latencies								
Global score	-0.3315	0.2685	**0.93**	**<0.0001**	0.2033	0.5053	0.5549	0.049
SEP	-0.6266	0.0219	**-0.7446**	**0.0035**	0.3791	0.2014	0.4231	0.1497
Path-Q	0.2328	0.4441	**0.935**	**<0.0001**	-0.163	0.5948	-0.6363	0.0194

Longitudinal analysis; neurophysiology

An incomplete dataset was available to me for the purposes of this thesis. Of the 29 participants of the study, the data for up to 16 was available from the last/fifth visit (V5). Numbers included in each of the analyses are indicated in the tables below.

Direct correlation of changes in the neurophysiological scores and changes in the EDSS over all time intervals did not show any consistent patterns of correlation or changes in those patterns over time *(Table 4.6*). Whilst there are some statistically significant correlations, notably for the global scores in the first year of the study, these are generally isolated findings. Of note, there are no significant correlations seen that include changes in disability at the end of the study (V5). This is in contrast to the cross-sectional data that suggested that correlation was greater in those who had had the disease for longer and/or were older.

In order to assess the predictive potential of changes in Neurophysiological parameters, correlation of the changes over 6 (V1-V2) *(Table 4.7)* and 12 (V1-V3) *(Table 4.8)* months with future EDSS (at V3, V4 and V5) and EDSS change from baseline at V3, V4 and V5 was calculated. Changes over the first 12 months seemed to be more informative then changes in the first 6 months only with regard to predicting future disability but again statistically significant results were sporadic. The changes appeared to more predictive of shorter term changes in disability (at V3 and V4) rather than those at the study end point (V5).

Table 4.6; Spearman's correlation coefficients (ρ/rho) of change in Neurophysiology score vs change in EDSS both from baseline (V1) and interval changes. 4-point ordinal scale and summed z-transformed latencies (as previously described) are both illustrated including subcomponents thereof. P-values of 0.05 or less are indicated in bold red, values of 0.01-0.05 are indicated in red.

		4-point ordinal scale				Sum of z-transformed latencies			
		Global	MEP	LL SEP	VEP	Global	MEP LL	SEP LL	VEP
V1-V2	**n**	21	21	21	21	**22**	22	22	22
	Rho	-0.115	0.043	-0.023	-0.103	**-0.361**	-0.178	-0.036	-0.048
	p	0.621	0.852	0.920	0.658	**0.099**	0.428	0.874	0.832
V1-V3	**n**	20	20	20	20	23	**23**	23	24
	Rho	0.501	0.514	-0.270	0.021	0.175	**0.625**	-0.099	-0.064
	p	0.025	0.021	0.250	0.932	0.425	**0.001**	0.655	0.766
V1-V4	**n**	18	17	18	18	20	20	20	20
	Rho	0.258	0.342	0.040	0.189	0.544	0.275	0.497	0.115
	p	0.301	0.179	0.874	0.454	0.013	0.240	0.026	0.628
V1-V5	**n**	12	12	12	12	13	13	13	13
	Rho	-0.0073	0.2354	-0.2648	0.0885	0.2264	0.0679	-0.1443	0.2292
	p	0.9821	0.4614	0.4055	0.7845	0.4570	0.8255	0.6380	0.4512
V2-V3	**n**	**20**	20	20	20	23	23	23	24
	Rho	**0.568**	0.409	-0.008	0.233	-0.052	0.239	0.188	0.156
	p	**0.009**	0.073	0.975	0.323	0.815	0.272	0.390	0.467
V3-V4	**n**	20	19	20	20	22	22	22	22
	Rho	-0.28	-0.25	-0.26	0.06	0.10	-0.20	0.16	-0.08
	p	0.23	0.31	0.26	0.81	0.64	0.36	0.49	0.72
V4-V5	**n**	14	13	14	14	15	15	15	15
	Rho	-0.040	0.087	-0.019	-0.432	0.091	0.001	0.050	0.373
	p	0.893	0.776	0.949	0.123	0.746	0.997	0.861	0.171

Table 4.7; Spearman's correlation coefficients (ρ/rho) of change in Neurophysiology scores from V1-V2 (0-6 months) vs EDSS both at timepoints V3, V4 and V5 and the change in EDSS from baseline at V3, V4 and V5. P-values of 0.05 or less are indicated in bold red, values of 0.01-0.05 are indicated in red.

		4-point ordinal scale				Sum of z-transformed latencies			
		Global	MEP	LL SEP	VEP	Global	MEP LL	SEP LL	VEP
V3 EDSS	**n**	24	24	24	24	25	25	25	25
	Rho	0.224	-0.010	-0.043	0.318	0.422	0.239	0.082	0.357
	p	0.293	0.962	0.842	0.130	0.036	0.249	0.698	0.080
V4 EDSS	**n**	21	21	21	21	22	22	22	22
	Rho	0.247	0.029	0.201	0.073	0.004	-0.076	-0.003	0.294
	p	0.281	0.900	0.383	0.753	0.986	0.737	0.989	0.184
V5 EDSS	**n**	16	16	16	16	16	16	16	16
	Rho	-0.0377	-0.1399	0.1873	0.5543	0.0857	0.0955	-0.0887	0.3459
	p	0.8898	0.6054	0.4872	0.0259	0.7524	0.7249	0.7438	0.1894
V1-V3	**n**	22	22	22	22	23	23	23	23
	Rho	-0.207	0.044	-0.203	-0.156	0.028	-0.099	0.196	-0.039
	p	0.355	0.847	0.366	0.489	0.899	0.653	0.371	0.861
V1-V4	**n**	19	19	19	19	20	20	20	20
	Rho	-0.079	0.162	0.074	-0.324	-0.291	-0.299	-0.095	-0.092
	p	0.748	0.508	0.765	0.177	0.213	0.200	0.691	0.698
V1-V5	**n**	14	14	14	14	14	14	14	14
	Rho	-0.412	-0.082	-0.049	-0.011	-0.572	-0.494	-0.496	-0.089
	p	0.143	0.782	0.867	0.970	0.033	0.073	0.071	0.763

Table 4.7; Spearman's correlation coefficients (ρ/rho) of change in Neurophysiology scores from V1-V3 (0-12 months) vs EDSS both at timepoints V3, V4 and V5 and the change in EDSS from baseline at V3, V4 and V5. P-values of 0.05 or less are indicated in bold red, values of 0.01-0.05 are indicated in red.

		4-point ordinal scale				Sum of z-transformed latencies			
		GEPS	**MEP**	**LL SEP**	**VEP**	**Sum-z**	**MEP LL**	**SEP LL**	**VEP**
V3 EDSS	**n**	22	22	22	22	25	25	25	25
	Rho	0.450	0.234	0.371	0.108	-0.014	0.108	-0.026	0.426
	p	0.036	0.294	0.089	0.632	0.947	0.608	0.903	0.034
V4 EDSS	**n**	**21**	21	21	21	22	22	22	22
	Rho	**0.680**	0.473	0.495	0.077	0.330	0.219	0.142	0.535
	p	**0.001**	0.030	0.023	0.739	0.134	0.327	0.530	0.010
V5 EDSS	**n**	16	16	16	16	16	16	16	16
	Rho	0.344	0.195	0.579	0.042	0.256	0.126	0.254	0.525
	p	0.193	0.469	0.019	0.878	0.340	0.643	0.342	0.037
V1-V3	**n**	20	20	20	20	23	**23**	23	23
	Rho	0.501	0.514	-0.270	0.021	0.175	**0.625**	-0.099	-0.063
	p	0.025	0.021	0.250	0.932	0.425	**0.001**	0.655	0.774
V1-V4	**n**	**19**	**19**	19	19	20	20	20	20
	Rho	**0.602**	**0.629**	0.069	-0.152	0.365	0.478	-0.077	0.112
	p	**0.006**	**0.004**	0.780	0.534	0.113	0.033	0.748	0.637
V1-V5	**n**	14	14	14	14	14	14	14	14
	Rho	0.297	0.341	-0.113	0.428	0.369	0.524	-0.096	0.064
	p	0.303	0.233	0.701	0.127	0.194	0.055	0.745	0.829

Discussion

In keeping with previous work in both the general MS population (Invernizzi et al., 2011; Leocani et al., 2006) and specifically the PPMS population (Schlaeger et al., 2014a) we have shown statistically significant cross-sectional correlation between evoked potential-based scoring methods and clinical parameters of disability. This is the largest cohort to date of interrogation of patients with PPMS, demonstrating marginally superior levels of correlation compared with earlier work.

In seeking to test for variation in correlation within the group, restriction of the dataset to patients who were either older or had experienced longer disease duration resulted in levels of correlation not before documented in the MS literature. The explanation for this finding is not immediately clear. When splitting the cohort according to age, the older group had a greater range of disability according to EDSS and a lower mean disability whereas when the patients were split according to disease duration those that had had the disease for longer had a narrower range of disability with an equivalent mean EDSS. In the only other comparably-sized study (Schlaeger et al., 2014a), whilst the highest levels of correlation were seen at the study endpoint, the lower numbers included in this data due to participant drop out makes the findings less robust, and the levels of correlation were not to the extent seen in this dataset.

Within-subject comparison of the relative strength of correlation of both neurophysiological and imaging parameters with disability measures has not previously been achieved in a PPMS cohort. The only imaging metric to correlate with the EDSS was the C5/6 cross sectional area with the strength of correlation inferior to that achieved with evoked potential-based scoring systems. This finding adds to the weight of evidence suggesting that evoked potentials, essentially a measurement of the function of eloquent neural pathways, shows greater promise in providing a surrogate for disability in PPMS than do established imaging outcomes.

The results of the longitudinal analysis are not all in keeping with previous study of the PPMS population. With the caveat of an incomplete dataset, the apparent disconnect between high levels of correlation in cross-sectional analysis and correlation in the changes of clinical and neurophysiological parameters over time is somewhat disappointing. Whilst statistically significant correlation of changes in the latency-based z-score and EDSS were seen over the first 2 years of the study, there is no pattern or consistency to the results and a loss of correlation at the study endpoint. This is in keeping with previous work which identified a 'trend' in longitudinal correlation without reaching statistical significance over a 3 year period (Schlaeger et al., 2014a). The predictive capacity of changes in Neurophysiological scores over the first 6-12 months was less than this study identified, though changes over the longer period seemed to provide more robust prediction, though still without pattern or consistency.

The results here suggest that z-transformed latencies are superior to ordinal scales and to categorical scores (giving mean number of abnormally prolonged EPs) as potential surrogates of disability within the PPMS population. This is likely to be due to the loss of data associated with categorisation of values in the ordinal scales. If these outcomes are to be translated to the clinical setting, regulator concerns regarding the meaning of output scores to the patient remain to be reconciled. As scoring methods increase in complexity and move away from single, simple, clinically relevant parameters the problem becomes more acute. Single parameter scores would be a move towards overcoming this conundrum, and in that domain measurements of the functionality of long-tracts would appear to provide the most hope. Previous work in the PPMS population has suggested that upper limb MEP and tibial SSEP could be used in place of a full 3 or 4 modal evoked potential protocol without losing substantial information (Schlaeger et al., 2014a); in this work however, the SSEP alone was equivalent to the globally-derived score in strength of correlation with EDSS. Whilst it does not measure a clinically identifiable outcome for the patient and does not involve calculation of a composite score, it does involve the dimensionless z-score. The inherent problems of using a single EP include the difficulty once electrical conduction within the pathway falls below a threshold and there is loss of output with no potential for further variability thereof.

The EDSS is heavily weighted towards mobility, being almost wholly dependent on ambulation beyond the threshold score of 4.0. Whilst there are some elements of

disability that are neglected or underrepresented in the scale, for the majority it provides a useful metric of the severity of disease. It is therefore not surprising that both neurophysiological and imaging parameters that measure the long tracts (both pyramidal fibres and sensory pathways that pass through the cervical cord) provide the most robust correlation with the EDSS. Given the historical use of the EDSS in prospective comparator populations, it remains the scale to which surrogate markers are likely to have to compare before use in clinical studies is ratified. Measures of the spinal cord and function of these long tracts are therefore likely to be central to the eventual solution.

Study limitations include significant variation in the disease duration at the time of recruitment, introducing structural weakness to data analysis and limiting the extrapolation and indeed to some extent the utility of the results. Further strengthening of findings could also have been achieved by imaging patients at the same time as recording the evoked potentials which would also enable assessment of direct correlation between imaging and evoked potentials. With regards to the longitudinal data there was some participant dropout and problems with missing data, likely a function of the duration of the study. Whilst neurophysiology is generally extremely reproducible, particularly with internationally accepted standards/minimum criteria, MEP does introduce a degree of operator variability and needs to be controlled for in future use. Finally, whilst DTI sequences were acquired in the MRI protocol these were not available to me for the purposes of this thesis. Preliminary work looking at correlation of fractional anisotropy with

disability in the general MS population has been promising and would certainly warrant future interrogation.

Future work should seek to test these correlations in significantly sized cohorts including those undergoing large scale therapeutic studies. Assessment of critically eloquent pathways that underscore motor disability warrant particular attention, with the potential for composite scores using both imaging and functional electrical studies – though with the caveat of difficulty in reconciling the concept with drug regulators, the ultimate goal of work of this nature.

Chapter 5

Conclusions

Whilst there has been significant progress in the efficacy and most prominently the mode of delivery of drugs that target increasingly specific inflammatory components critical to the development of MS over the last 2 decades, the disease remains far from a curable condition. Furthermore, potent immunosuppression even of very specific targets within the immune system is not achieved without significant risk of potentially fatal complications. The disconnect between inflammation and the neurodegeneration that ultimately underlines disability has been widely discussed and continues to limit the success of increasingly potent immunosuppressants.

Neuroprotection, immunomodulation and cell replacement, all demonstrable properties of adult multipotent mesenchymal stem cells offer further therapeutic potential that may help to address not just the inflammatory disease component but also the critical neuronal attrition that underscores patient disability. Much work has been done over the last decade and more to elucidate these properties; with the accumulation of a critical body of evidence, translation to clinical trials is underway.

Animal models of inflammatory neurological pathology have yielded many clues to the mode of activity of adult stem cells, predominantly MSCs. Detailed knowledge of their location/mobilisation, function and properties in man however is lacking. Whilst there are many similarities between EAE and MS, there remain critical pathophysiological differences limiting extrapolation from laboratory work to the clinic and prompting some of the work described in this thesis.

Mobilisation of haematopoietic stem cells in the context of anti-CD49d activity has been demonstrated and may represent an unrecognised therapeutic contribution in its clinical use. Alongside replicating these earlier findings, we have here shown that cells expressing the same marker are present in the circulation in higher numbers in patients with MS undergoing clinical relapse than those in remission. We have also demonstrated the presence of release of the $CD133^{+}$ subset (with greater proliferative and differentiation potential) within this population of cells, in those undergoing relapse.

It is in some respects unsurprising that cells with regenerative capacity are released as part of the inflammatory cascade that is so prominent in MS but this has not before been demonstrated. The precise therapeutic contribution of these cells in MS in humans is unknown, but the regenerative potential of the cells *in-vitro* as well as *in-vivo* (in the context of animal models) is well documented, including the potential for regenerative contribution of haematopoietic stem cells to non-haematopoietic tissues.

Alongside identification of haematopoietic stem cells in peripheral blood samples we also attempted to isolate stromal stem cells in the form of multipotent mesenchymal stem cells. Whilst the detection of these cells within the bone marrow niche was readily achieved using a novel cytometric algorithm, detection in the peripheral blood stream where they are present in lower numbers proved challenging, including in subjects in whom other stem cell subsets had been mobilised either in the context of relapse or administration of an endogenous cell mobilising agent.

With time, inevitable technological progress in rare event detection using multichannel cytometric techniques will enable more robust identification of these circulating cells. Clearly this will have significant therapeutic utility in enabling indirect monitoring of the intra-venous infusion of MSCs for a wide range of both neurological and non-neurological conditions. Direct evidence for the dynamic monitoring and eventual fate of intra-venously delivered MSCs is likely to depend upon the development of labelling technology that is firstly safe in human use, and secondly enables identification by localisation techniques at the single cell level, a highly technically challenging objective.

Additional evidence to support the theory of multipotent mesenchymal stromal cell mobilisation and contribution to the pathophysiology of restorative processes in the context of MS, comes from identification of cells expressing relevant cell markers within lesions in MS-affected brain tissue. Consistent with the identification of subsets of stem cells released during periods of inflammatory

activity, these cells were more readily identified in active lesions in patients with progressive disease - either primary progressive disease or following an earlier relapsing- remitting course. The cells in question were found in parallel with the inflammatory infiltrate of activated microglia both throughout active lesions and at the 'active' rim at the periphery of chronic active lesions. None were identified in the brains of control subjects who did not have neurological or systemic inflammatory pathology.

The difficulty in identifying cells with no single defining characteristic and in whom cell surface molecule expression varies according to lineage commitment is self-evident. Nonetheless, the dual expression of at least two proven markers, in tandem with the absence of markers of the alternative cellular components of white matter brain tissue, provides not insignificant evidence to support this hypothesis. Whilst the level of infiltration of areas of disease activity within the CNS of patients with MS was greater than might have been expected, this is not something that has previously been explored in the neuroscientific literature, and is not out-with the realms of pathophysiological possibility. The ability of these cells to home to sites of tissue injury is well established, as are a multitude of properties that have protective and restorative capacity. Indeed it is hypothesised that, contrary to the traditionally held view of supportive stromal function within the bone marrow niche, the principal role of these cells is to protect and repair tissue throughout the human body. It is therefore in many ways of no surprise whatsoever that they are found in the damaged brains of people with a neuroinflammatory condition.

The ability to detect therapeutic gain has long challenged those working within the field of multiple sclerosis, as in many other neurodegenerative conditions which progress over many years. Whilst the inflammatory component of the disease is relatively easy to quantify, measure and record, we have discussed how its fundamental relationship to progressive disability is at best questionable. The recent development of the outcome measure of 'no evidence of disease activity' (both clinically and radiologically) makes attempts to circumvent some of these problems but again remains purely a marker of inflammatory activity.

We have shown that Neurophysiological markers of CNS integrity/function are superior to established imaging metrics as a surrogate marker of disability as measured by the EDSS in primary progressive MS. Whilst the EDSS is an imperfect cornerstone on which to base these judgements, historical use of the EDSS and the practical difficulties associated with contemporary natural history studies means that it will necessarily remain central to such endeavours.

High levels of correlation between the EDSS and scores based on evoked-potential neurophysiological parameters were identified in cross-sectional analysis, with particularly high levels seen in patients who were older and had had the disease for a longer duration. Whilst changes in the evoked-potential based scores did not correlate systematically with changes in the EDSS, there were areas of promise. The use of z-transformed latencies which continue to capture deterioration of neuronal pathways up to the point of conduction failure would appear to represent the most

promising measure for the purposes of detection of disease progression in established disease. Whilst the correlation over the longest intervals was disappointing, this may have been a factor of a reduction in sample size due to participant drop out and an incomplete dataset.

The ideal method of scoring the neuronal dysfunction captured in neurophysiological assessment remains to be definitively established. Any developments that enable streamlining of the acquisition of representative data would clearly be most helpful but maintaining relevance to the patient and acceptability to regulators as a viable disease outcome measure remains the most crucial element still to be reconciled.

Future directions for the work described in this thesis include further refinement of algorithms to detect MSCs within the bloodstream. Ultimately this is likely to depend upon technological progress as well as the design of an appropriate algorithm before the goal is achieved. Further evaluation of the inflammatory profile of the cell release in acute relapse and their correlation with the speed and degree of recovery may prove instructive in further identifying advantageous cell populations and refining therapeutic use. Lastly, utilisation of larger datasets (including those that are acquired from the planned 100 patients entering the ongoing phase II study of autologous bone marrow transfer in relapsing-progressive MS in Bristol) to strengthen a number of primary findings in smaller pilot studies such as that described in this work.

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